Cell differentiation and adhesion in colorectal cancer

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promotores
Prof. dr. J.W. Arends
Prof. dr. F.T. Bosman (University of Lausanne, Zw.)

Co-promotor
Dr. J. ten Kate (De Weverziekenhuis, Heerlen)

Beoordelingscommissie
Prof. dr. F.C.S. Ramaekers (voorzitter)
Prof. dr. H.F.P. Hillen
Prof. dr. M. Mareel (Rijksuniversiteit Gent, B.)
Prof. dr. M.F. von Meyenfeldt
Prof. dr. D.J. Ruiter (Katholieke Universiteit Nijmegen)

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1.1 Introduction

Colorectal cancer is one of the most common forms of cancer in western countries. The annual incidence is second only to lung cancer in males and to breast cancer in females[1,2]. In Europe the annual mortality rate reaches 80.000, in the Netherlands alone 7500 new patients are diagnosed every year[1]. Like in most solid tumours despite more intense treatment these figures have not substantially improved over the past two decades.

Treatment of choice still remains radical surgery with adequate margins of colon and removal of regional lymph nodes[3-5]. However, even after curative surgical resection, 5-year survival is in the order of 30-50%[6,7]. Chemo- and radiotherapy have not dramatically changed survival[8], although for Dukes C adenocarcinomas adjuvant chemotherapy (Fluorouracil and Levimasole) results in a reduction of the mortality rate to 33%[9].

The two most important determinants of prognosis are stage and grade assessed by pathologists. Stage is defined by the process of invasion and metastasis, grade upon the differentiation of the tumour cells.

Tumour stage is based on the Dukes classification[10] which describes the stage of extension of the adenocarcinomas. In this classification Dukes A is defined as extension of the tumour into the submucosa or muscularis propria and Dukes B as tumour growth through the bowel wall without metastasis. Dukes C tumours in addition show lymph node metastasis, while Dukes D[11] tumours grow into adjacent organs and/or show distant metastasis. Throughout the years, different modifications of the Dukes classification were introduced, such as the Astler-Coller staging system[12] and the TNM classification of which a schematic overview is presented in Table I. Staging of tumours only provides prognostic information at the population level. It should be realized that at the individual level chances of survival of patients within the same stage may substantially vary[5].

Grading is much less relevant for prognostication than staging, but at the population level well, moderately well and poorly differentiated carcinomas divided according to architectural and cytonuclear features[13] show slight differences in respective survival. Histological grading, however, is subjective and great intra- as well as interobserver variations have been reported. Other histological features which have been evaluated as prognostic parameters include eosinophilia[14] and the extent of the inflammatory infiltrate as a reflection of the host respons to the tumour. Jass et al.[15,16] combined tumour growth pattern and grade features in his staging system (see also Table I).

Another way to assess tumour grade has been to analyse differentiational characteristics of the tumour cell population through the expression of markers of differentiation which have been developed for the different cell lineages in the

Table I.

Dukes	A	extension of the tumour up to muscularis propria
	B1	into muscularis propria
	B2	through muscularis propria, but limited to the bowel wall
	B3	through muscularis propria, through serosa
	C1	up or into muscularis propria, without lymph node metastasis
	C2	through muscularis propria, with lymph node metastasis
	D	into adjacent organs and/or distant metastasis
Astler-Coller	A	mucosal involvement only
	B1	into muscularis propria
	B2	through muscularis propria
	C1	B1, with lymph node involvement
	C2	B2, with lymph node involvement
	T1	tumour growth into submucosa
	T2	into muscularis propria
	T3	into subserosa, non-peritonealized pericolic tissue
	T4	into other organs or structures
	N1	three or less lymph nodes
	N2	more than three lymph nodes
	N3	nodes on named vascular trunk/ apical nodes
	M	distant metastasis

Jass tumour staging	Scoring
Limitation of growth to bowel wall	
Yes	0
No	1
Invasive margin	
Expanding	0
Infiltrating	1
No. of lymph nodes with metastasis	
0	0
1-4	1
>4	2
Conspicious peritumoural lymphocytic infiltrate	
Yes	0
No	1

Scoring: Group I, 0-1 (excellent prognosis); Group II, 2; Group III, 3; Group IV, 4-5 (poor prognosis)

colonic epithelium identifying end stages of differentiation. For these markers a consistent prognostic significance has not been reported[17], whereas these often appear to be coexpressed in a large proportion of the tumours[18]. An exeption, however, seems to be neuroendocrine differentiation, which was repeatedly found to correlate with worse prognosis[19-21]. Other investigators, however, reported no prognostic significance for neuroendocrine differentiation[22].
The disappointing and inconsistent contribution of the available histological parameters to the establishment of prognosis forms the background of this study. We reasoned that in search of markers for tumour behaviour, in stead of parameters related to terminal tumour cell differentiation it might be more worth while to look for stem cell characteristics since these cells are responsible for tumour growth, invasion and metastasis.
After a brief discussion of the molecular events in colorectal carcinogenesis in this introductory chapter, we will develop the stem cell concept in relation to differentiation in colon cancer and also discuss the role of cell adhesion in colorectal cancer metastasis.

1.2 Invasion and metastasis in colorectal cancer

The process of metastasis is divided into sequential steps[23,24]. First, the carcinoma cell has to detach from the primary tumour probably by downregulating the expression of specific cell-cell adhesion molecules[25,26]. Secondly, to traverse the epithelial basement membrane, enhanced proteolytic activity is needed[27]. Also, cell-basement membrane adhesion molecules have to be inactivated[28]. In order to traverse the extracellular matrix, motility factors should be expressed[29-31]. Thirdly, the barrier of lymph- or blood vessel wall has to be taken for further dissemination and the carcinoma cells will have to survive in the circulation. The pattern of metastatic spread probably is partly determined by site specific interactions with organ specific endothelial cell properties and by tissue specific microenvironment. Finally, the invasion process is reversed: traversing of the vessel wall and extracellular matrix occurs and at the metastatic site again cell-cell adhesion molecules are expressed to reestablish tissue architecture, finally resulting in a metastatic tumour.

1.2.1 Molecular carcinogenesis

The paradigm for molecular carcinogenesis of colorectal cancer is the Vogelstein concept. A cascade of molecular events has been proposed in which to some extent the sequence, but more importantly the accumulation of a number of genetic abnormalities determines the final biological behaviour of the tumour[32-35].

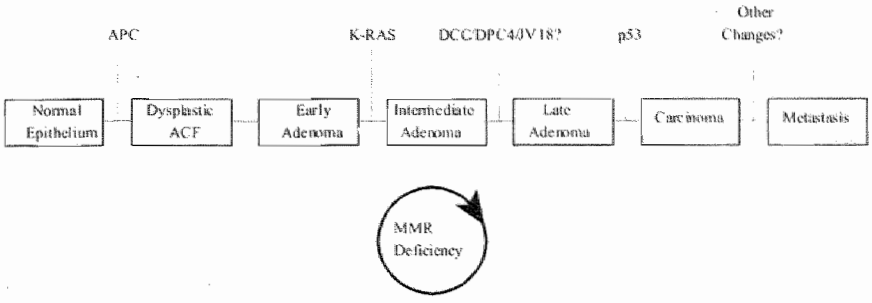

Figure 1. Genes in colorectal carcinogenesis

The different onco(suppressor) genes playing a role in colorectal carcinogenesis are shown schematically in Fig. 1.

It is beyond the scope of this thesis to extensively discuss the individual genes, but APC and DCC are of interest given the context of our investigation.

The APC (Adenomatous Polyposis Coli) tumour suppressor gene locus was discovered by identification of a constitutional deletion of the chromosomal band 5q21 in a patient with familial adenomatous polyposis[36]. The gene was identified by positional cloning. In colorectal cancers with no known familial predisposition, allelic loss of chromosome 5q21 has been found in 20-50% of the cases[33]. 5q allelic losses are most frequently detected in small early adenomas from patients without polyposis, which suggests that inactivation of one or both of the alleles by somatic mutation occurs at an early stage of tumour development. APC is considered to be the gate keeper gene in colorectal tumourigenesis. The gene product is a large protein (a 2844-amino acid polypeptide) with different domains binding to several other proteins, amongst which β-catenin, which is involved in cell adhesion through its complexion with E-cadherin[37,38]. Recently it has become apparent that β-catenin also plays a role in the Wnt signaling sequence through T cell transcription factor 4 (Tcf-4)[39-41]. APC also colocalizes with the tubuline skeleton and is claimed to be involved in the process of apoptosis[42].

The DCC (Deleted in Colorectal Cancer) gene was also identified by positional cloning and is located on chromosome 18q21. Loss of heterozygosity (LOH) at this locus occurs in 70% of carcinomas and in 50% of far advanced adenomas, which made this region suspect for localization of a tumour suppressor gene[33]. Also, LOH of 18q has been correlated with poor prognosis[43]. The DCC gene encodes a protein with homology to neural cell adhesion molecule, NCAM[44].

For this reason it is conceivable that the DCC product is involved in invasion, altered adhesion and metastatic potential of tumour cells.
Despite the unraveling of some genetic factors associated with colorectal tumourigenesis, the exact mechanisms leading to colon carcinoma, whether genetic or environmental largely remain unknown. Vogelstein et al. postulate in their model that once carcinomas are formed, the accumulated loss of suppressor genes correlates with the ability of the tumour to metastasize. However, no detailed genetic alterations have been described in the association of metastasis. For prognosis, however, this process of metastasis largely determines patient survival.

1.2.2 Cell adhesion molecules

In invasion and metastasis, cell adhesion molecules (CAM) play a pivotal role. Invasive cells must detach from their intercellular connections, which are mediated by CAM, and need to develop a surface molecular structure, including the expression of matrix receptors, which allows them to interact with the extracellular matrix. (For an overview see Freemont[44]).
Adhesion molecules generally are divided into five families: selectins, immunoglobuline-like superfamily, integrins, hyaluronate receptors and cadherins (Fig. 2).

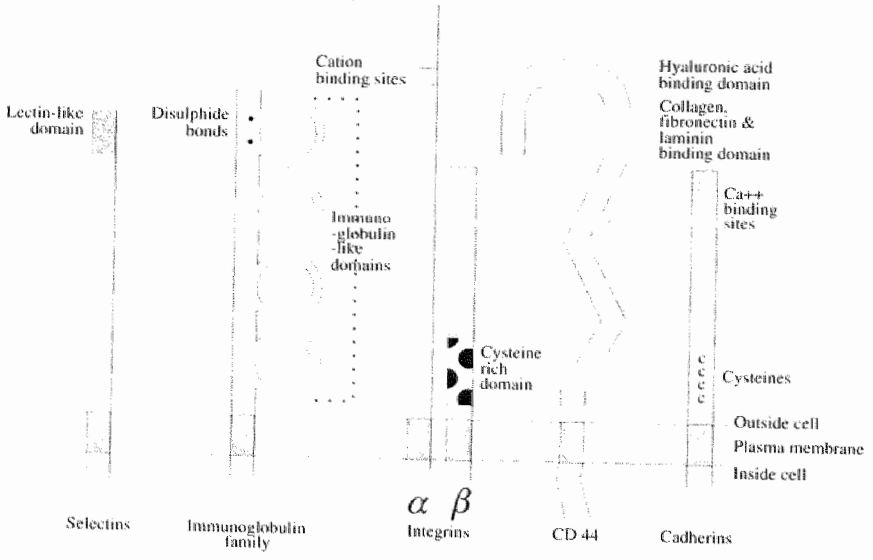

Figure 2

A Selectins. The selectin molecules possess three different protein domains; a calcium dependent lectin domain, a repeat domain corresponding to that in epidermal growth factor (EGF) and a varying number of repeats of a domain of complement regulatory proteins (CRP). There is a high degree of homology between the selectins, which is probably the result of duplication of an ancestral gene, followed by exon diversification and duplication. The genes for all selectin proteins are on chromosome 1.

Selectins play an important role in the inflammatory response by taking part in the earliest phases of leucocyte extravasation. Leucocytes move towards the wall of the capillaries and begin to role along the endothelium[45,46]. This process requires interaction between a receptor on endothelial cells-the selectins- and a ligand on the moving leucocytes (on neutrophils, the ligand for the selectins is the sialyl Lewis X determinant).

The selectin family has three members: E-, P- and L-selectin. E-selectin is expressed on endothelium and mediates neutrophil, monocyte and some T lymphocyte adhesion to the endothelium. E-selectin may play a role in metastasis since it was shown to mediate binding of the colon carcinoma cell line HT-29 to endothelium[47]. Also, the ability of cell lines to bind to E-selectin was shown to be directly related to their potential to metastase to the liver[48]. L-selectin is expressed on peripheral T and B lymphocytes, neutrophils and bone marrow derived cells. Apart from a role in the process of leucocyte rolling, this selectin also functions as a homing receptor. P-selectin is expressed on thrombocytes and endothelium and mediates adhesion of neutrophils and monocytes to thrombocytes and endothelial cells, thereby causing a histamin release few minutes after thrombin activation. Also, interaction of activated platelets with tumour cells via P-selectin may facilitate metastasis through arrest of the formed aggregate in microvessels or by the provision of platelet growth factor to the co-aggregated tumour cells[49].

B Immunoglobuline-like superfamily. The Immunoglobulin-like superfamily of adhesion molecules consists of more than seventy members. All members have their molecular structure in common with the immunoglobulin-fold, consisting of two β pleated sheets stabilized by disulfide bonds, as basic common motif. Members include molecules concerned with antigen recognition by and adhesion to lymphocytes (CD3,CD4). Another subgroup is formed by the intercellular adhesion molecules (ICAM), which are expressed on epithelial and endothelial cells. Also included are the neural cell adhesion molecules (NCAM)[50]. As mentioned before, the DCC gene codes for a protein with homology to NCAM[34].

C Integrins. Integrins are a family of heterodimeric membrane glycoproteins expressed on a diversity of cell types which function in cell-cell adhesion as well as

cell-substratum adhesion. Their structure is very characteristic and formed of two non-covalently associated subunits, α and β. Initially the integrins were subdivided into three subfamilies (β1 or Very Late Activated VLA proteins, β2 or leucams and β3 or cytoadhesins), in which a β unit was thought to associate with different α units. However this classification has become less rigid since more α than β units have been characterized nowadays and individual α units can associate with more than one β subunit. The recognition site for some integrins is an RGD sequence (Arginin, Glycin and Aspartic acid) on the extracellular matrix. In vitro, RGD peptides have been shown to inhibit tumour cell migration[51,52].

D Hyaluronate receptors. Hyaluronate is a component of the extracellular matrix and plays a role in several pathological processes including inflammation and carcinogenesis. Cells bind to hyaluronate through cell surface proteins, one of which is CD44. Through alternative splicing of its exons, CD44 can display heterogeneity with a variety of physiological roles for the splice variants including cell migration. A specific isoform implicated in metastatic potential is called CD44v. In colonic cancers, a direct relationship has been described between this isoform and metastatic potential: the isoform CD44v6 is expressed in all cases of the most aggressive tumours, whereas in normal colonic epithelium no expression is found[53]. In addition, it has been shown that CD44 variant expression has prognostic significance in colorectal cancer[54].

E Cadherins. The Cadherin family is made up by calcium dependent homotypic cell adhesion molecules playing a role in the maintenance of integrity of multicellular structures. The molecules are also important in embryologic development[55]. They consist of a precursor polypeptide which after a series of post-translational modifications (glycosylation, phosphorylation and proteolytic cleavage) becomes a mature protein of 723-748 amino acids. In the extracellular domain some repeats contain a calcium binding site. Also a transmembrane and a short cytoplasmic domain with a highly conserved region of homology exist. Until now many different cadherins have been identified amongst which E-(epithelial), N-(neural), and P-(placental) cadherins are the best known. The first detected cadherin was called uvomorulin, because it was found to play a role in early mouse development where it mediates the compaction process at the morula stage[56]. E-cadherin is also known as cell-CAM 120/80 and is homologous to L-CAM in the chicken and Arc-1 in the dog[57,58]. The cadherins are concentrated at the zonulae adherentes (cell-cell junctions) of epithelial cells and are associated with actin of the cytoskeleton via cytoplasmic proteins called catenins. Three catenins have been described: α, β and γ catenin with a molecular weight of respectively 102 kD, 88 kD and 80 kD. Binding of the cadherins via

catenins is essential for cadherin function[59-61]. As outlined before, the APC gene product is also involved in this complex through binding with β-catenin[47,48].
The expression of E-cadherin in carcinomas has been studied extensively. In human tumour cell lines E-cadherin was found to act as an invasion suppressor[62]. It has also been shown that phosphorylation of E-cadherin-catenin complex increases invasiveness in vitro[63].

1.3 Differentiation

1.3.1 Normal colonic epithelium, the different cell lineages

The colonic mucosa consists of crypts of Lieberkühn lined with a single layer of epithelium. The crypts are embedded in a loose stromal tissue and reach to the muscularis mucosae, which separates the mucosa from the underlying submucosa.
The lining epithelium shows three types of cells: columnar shaped absorptive enterocytes, mucin producing goblet cells and neuroendocrine cells. In case of inflammation and (pre)cancerous change, a fourth cell which normally occurs primarily in the small intestine is present, the lysozyme producing Paneth cell.
Columnar cells have an important function in absorption of metabolites, for which they have microvilli at their luminal surface. Differentiated columnar cells synthesize secretory component, which is a glycoprotein with a molecular weight of 60-70 kD serving as a carrier in the epithelial translocation of dimeric IgA, which is subsequently secreted into the bowel lumen in defense against microorganisms. Antibodies against secretory component are available to stain the differentiated columnar cells[64,65].
Goblet cells secrete mucin for lubrication purposes and also to protect the epithelium against mechanical damage and chemical irritants. Colonic mucus is a molecular mixture in which sulfomucins predominate, but admixed with neutral and sialomucins and which can be stained with convential techniques such as PAS, Alcian Blue and HID-AB stains. Also different monoclonal and polyclonal antibodies can be used for immunohistochemical detection of mucin.
Neuroendocrine cells synthesize polypeptide hormones and through these modulate intestinal functions such as secretion, absorption and motility. These cells can be recognized by monoclonal antibodies against the different polypeptide hormones or with monoclonal antibodies which recognize compounds in neurosecretory granules such as Chromogranine A. Also, histochemical techniques, amongst which silver impregnations are most widely used, have been developed[66,67].
Paneth cells produce lysozyme, an enzyme capable to cause lysis of bacterial cell walls. Normally this cell can be found in the small intestine at the bottom of the

crypt. During inflammation and (pre)cancerous change, these cells are seen in colonic epithelium. Antibodies against lysozyme are available to stain Paneth cells.

As stated before, the markers associated with terminally differentiated cells, perhaps with exception of neuroendocrine markers, do not provide essential information about the clinical behaviour of colon tumours, probably because -also in a tumour- such differentiated cells are destined to go into programmed cell death and do therefore not contribute to tumour growth and progression. Progenitor cells or stem cells, in contrast, are the cells that propagate and therefore determine the fate of tissue, whether normal or malignant. Against this background markers of such cells might be more meaningful in the assessment of the clinical course and behaviour of tumours.

1.3.2 Colonic stem cells

For colonic mucosa, the presently held views regarding epithelial cell maturation were originally formulated by Cheng and Leblond[68], who provided evidence that all cell types are derived from the same precursor cell, which they postulated to be the crypt stem cell. The stem cell is located at the bottom of the crypt and gives rise to daughter cells with proliferative potential. During movement upwards to luminal crypt position, proliferative capacity is lost and gradual differentiation takes place. Finally mature cells are shed into the bowel lumen. This so called unitarian theory was subsequently corroborated by experi-

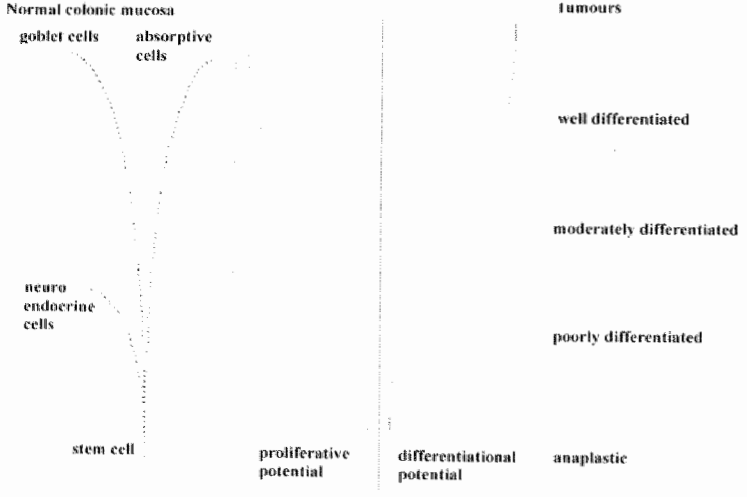

Figure 3. Different stages of malignant transformation

mental embryological studies, using the chicken-quail neural crest transplantation technique[69]. Recent studies by Park et al. also support the stem cell concept[70].

1.3.3 Differentiation and maturation of tumour cells. The tumour stem cell concept

It is conceivable that in analogy with normal mucosa, tumours arise from a single (tumour) stem cell (Fig. 3). The population of tumour cells is indeed monoclonal[52]. Several observations support the validity of the stem cell concept for neoplasms. Firstly, several case reports mention a wide spectrum of differentiation in adenocarcinomas[71,72]. This could reflect the idea that these tumours originate from a pluripotent stem cell. Secondly, differentiation induction in colon cancer cell lines leads to reduced proliferative capacity[73-77], suggesting that only stem cells propagate and differentiated cells do not contribute to tumour growth. Thirdly, studies on subpopulations of cells from human tumours have shown that these may show a variable degree of proliferative capacity, clonogenic potential and cell differentiation[78-81]. Finally, in clonogenic studies cloning efficiencies of tumour cell populations as low as 0.001% are generally found[82,83], suggesting that the proliferating tumour cell fraction in human tumours is small and could only be derived from a stem cell fraction.

The basic principle of the tumour stem cell concept is that a maturation arrest occurs at the moment of malignant transformation of a cell into a tumour cell. According to this concept the behaviour of a tumour reflects the proliferative and differentiational potential of the cell, from which the tumour originates: when this transformed cell in its phase of development was stem cell like, the resulting tumour would be rapidly growing and poorly or undifferentiated, whereas in contrast a tumour arising from a differentiated cell would be slowly progressing and differentiated. Fig. 3 depicts the variations to be expected in the spectrum of colorectal tumours according to proliferation and differentiation. From this concept it is obvious that it is of paramount importance to search for stem cell markers since these characterize highly malignant, metastasizing tumours. Therefore, markers for stem cells or "near" stem cells, and immature/not terminally differentiated cells of the various lineages (columnar, goblet and neuroendocrine) should be generated.

1.4 Aim

Colorectal carcinomas can be cured by complete excision of the tumour. Therefore, surgery is still the treatment of choice. Treatment failure is thought to be caused by (minimal) residual tumour. It is essential, therefore, to identify tu-

mours with high chance for incomplete removal. Aggressively behaving tumours, probably early metastasizing tumours, are the ones responsible for residual tumour and treatment failure.
In this context the aim of this study was to contribute to the work of pathologists to better evaluate the clinical course and behaviour of especially very aggressive and metastasizing colorectal tumours. This was approached by two ways: 1) generation of markers of immature/stem cell(like) cells to hopefully enable the identification of tumours with a highly malignant prophile and behaviour and 2) study the expression of some essential molecules in the process of cell adhesion so as to possibly identify tumours with a metastasizing potential.
Chapter 2 detailedly describes our efforts to generate monoclonal antibodies reacting to antigens related to immature or stem cell (like) cells. The characteristics of one of the obtained monoclonal antibodies are extensively reported in chapter 3.
Chapter 4,5 and 6 relate our experience with the studies of adhesion molecules. In chapter 4 the question is addressed whether invasive potential of primary tumours could be inferred from loss of E-cadherin expression. The hypothesis that it could be possible that metastases arising from cells deficient in adhesion molecule expression also show reduced E-cadherin expression was studied in chapter 5. Finally in chapter 6 the impact of the cadherin-catenin complex in metastatic behaviour was investigated.

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Chapter 2

Colonic crypt isolation and production of anti-crypt cell antibodies

Anna A.M. van der Wurff, Winand N.M. Dinjens, Edith P.M. van der Linden, Patrick T.J. Marx, Joop ten Kate, Jan-Willem Arends and Fred T. Bosman.

Introduction

In order to obtain monoclonal antibodies specific for a defined molecule or a topographically or functionally defined tissue structure either a well defined antigen has to be used in the process of immunization or a suitable test system has to be used to select the antibody with the desired specificity subsequent to immunization with an ill defined immunogen. In the setting of our desire to obtain crypt stem cell specific antibodies this implied either the availability of crypt stem cell specific antigens or a test system which would allow us to select for crypt stem cell specific immunoreactivity. Crypt stem cells in the colon are not well defined and therefore antigens specific for these cells are not available. In principle, immunohistochemistry would be the method of choice for selection of appropriately immunoreactive hybridomas, as crypt stem cells are topographically defined. In practice, common crypt cell antigens are so dominant that it would require an inordinate effort to select an antibody by immunohistochemistry, following immunization with unselected crypt cell antigens. We therefore set out to eliminate common crypt cell antigens during or prior to immunization, in order to limit the spectrum of obtained immunoreactivities. To reach this goal the following approaches were chosen:

1) selection of specific crypt cell subpopulations. This was done by differential harvesting of enzymatically digested colon mucosa and by cell culture techniques, using cell lines in different stages of maturation.

2) antibody blocking of common crypt cell antigens and

3) in vivo selection of the immune response using cyclophosphamide.

The antigenic mixture was injected intrasplenally into mice, because in this way we hoped the immune system would even recognize weakly immunogenic antigens.

In this chapter the results of these efforts are described.

Materials and methods

Selection of specific crypt cell subpopulations

The problem of antigen selection was approached in two ways. First, we attempted to isolate the basal crypt cells to be used for immunization. In addition, differentiated and undifferentiated colorectal cancer cell lines were used as antigenic mixtures.

Crypt base cell isolation: Human colonic crypts were isolated from surgical colectomy specimens according to Whitehead et al.[1]. Normal looking mucosa at least at five cm distance from the tumour was excised and placed in a sterile petridish containing 1 mmol/l EDTA, 1 mmol/l ethyleneglycol-bis-(B-aminoethyl

ether)-N,N,N,N-tetraacetic acid (EGTA)(Sigma chemical company, St. Louis, MO) and 0.5 mmol/l DTT in PBS (Phosphate Buffered Saline pH=7.4) (30 min RT). Next the tissue was put in a conical centrifuge tube with sterile PBS and shaken vigorously by hand. The crypts liberated by this procedure were harvested by gentle centrifugation (g=100) and lysed with lysis buffer containing 5 mmol/l $NaHPO_4$, 1 mmol/l EDTA, 1 mmol/l β mercaptoethanol, 0.125% m/m deoxycholate, 0.125% v/v Triton X-100, 250 mmol/l sucrose (pH=6.4) mixed with protease inhibitors (1 mmol/l PMSF, 2 mmol/l NEM).
In order to obtain the lower part of the colonic crypt epithelium, including the crypt base stem cells, the mucosa was incubated, after decontamination with 0.04% m/v Natriumhypochloride in PBS (15 min, RT), with 0.05 mmol/l EDTA in CMF-HBBS (30 min, 37°C) in a spinner flask (Gibco) according to Ten Kate et al.[2]. This supernatant yields single, more luminally located epithelial cells. The mucosa with the remaining basally located epithelium was subsequently incubated as described above to yield crypt bottom parts.
Cell culture: In our immunizations we also used cells from the colon cancer cell lines HT-29, $CaCO_2$ and NCI-H716. HT-29 and $CaCO_2$ can differentiate in vitro; NCI-H716 is an undifferentiated cell line. In order to maintain HT-29 cells undiferentiated, galactose instead of glucose was added to the medium (DMEM supplied with 5% FCS)[3]. $CaCO_2$ cells remain undifferentiated in exponential growth. NCI-H716 cells grow in suspension and do not differentiate in vitro[4].

Immunization

Immunization was done by injection of antigens directly into the spleen of Balb/c mice, thus, we reasoned, enabling the immune system of the mouse to recognize even weakly immunogenic antigens. To avoid rupture of the spleen, the mice recived one injection into the spleen 72 hours prior to fusion.
Mice were immunized with lysates of undifferentiated cells from cell lines and from human colon tissue. Also specific approaches like blocking of common crypt cell antigens and in vivo selection of the immune respons were used.
Blocking of common crypt cell antigens: As antigenic mixtures, undifferentiated $CaCO_2$ cells, undifferentiated HT-29, NCI-H716 cells and crypt bottom epithelium was used. These mixtures were lysed in buffer containing 5 mmol/L $NaHPO_4$, 1 mmol/L EDTA, 1 mmol/L β-mercaptoethanol, 0.125% (m/m) deoxycholate, 0.125% (v/v) Triton X-100, 250 mmol/L sucrose (pH=6.4) mixed with protease inhibitors (1 mmol/L phenylmethylsulphonyl fluoride (Sigma), 2 mmol/L N-ethylmaleimide (Sigma). To block common crypt cell antigens, the lysate was repeatedly mixed with antisera against colonic epithelium generated in mice (1 ml lysate was mixed with 40 µl undiluted antisera) and with mono-

clonal antibodies against common antigens (50 µl spent hybridoma culture medium) i.e. the anti-mucin antibodies Parlam 9 and 10[5]. Bound antibodies were precipitated with Sepharose protein A (Pharmacia). 100 µl of the remaining supernatant was injected into the spleen of Balb/c mice.

In vivo selection of the immune response: Ten minutes after intraperitoneal administration of differentiated HT-29 or $CaCO_3$ cells or differentiated human colon epithelial cells harvested after 30 minutes of cell isolation[2], mice were injected with cyclophosphamide (100 mg/kg) given intraperitoneally in saline. The same dose of the drug was administered at 24 and 48 hours. The same cyclophosphamide administration scheme was repeated every two weeks until after three treatments mice were bled from their tails. Sera were tested by immunohistochemistry on sections of small and large human intestine for the presence of polyclonal antibodies and weak immunoreactivity of sera of mice immunized with cyclophosphamide treatment was noted in comparison to sera of mice without cyclophosphamide treatment. A final boost with undifferentiated HT-29 cells, $CaCO_3$ cells or undifferentiated crypt bottom epithelial cells was given 72 hours prior to fusion. This immunizaton protocol is drawn schematically in Table I.

Fusion protocol

30 Fusion was done according to Köhler and Milstein[6]. In short, spleen cells were fused with Sp 2-0 myeloma cells using PEG 4000 (Merck, Darmstadt) in a ratio 2:1, seeded in 96 well microtiter plates and grown on DMEM, 20% FCS, 2% HAT (Boehringer, Mannheim), 2% penicilline and streptamycine, 2% glutamine and 1% pyruvate. Hybridomas were cloned by limiting dilution.

Table I.

Immunizaton (t=0) with *differentiated* cells.

t=10 min	cyclophosphamide 100 mg/kg intraperitoneally
t=24 hrs	id
t=48 hrs	id

Repeat every two weeks.
Final immunization (72 hrs prior to fusion) with *undifferentiated* cells.

Antibody selection

Sections of ethanol fixed and paraffin embedded normal large and small intestinal epithelium were made, each piece placed at such a distance that 5 consecutive sections could be placed on one glass slide[7]. Supernatant of all 96 wells of each microtiter plate was tested by an indirect immunoperoxidase method and hybridomas producing antibodies with a differential staining pattern were cloned and recloned by limited dilution to obtain monoclonal antibodies.

Results

Selection of specific crypt cell subpopulations

We succeeded to preferentially isolate crypt base cells with the method of Whitehead et al.[1]. Combined with the method of Ten Kate et al.[2] also superior crypt cells could be isolated. Also cultured cells including differentiated and undifferentiated HT-29 and $CaCO_2$ cells and undifferentiated NCI-H716 cells were used as antigens.

Antibody selection

Immunization had to be performed with complex antigen mixtures because antigens of interest i.e. antigens of undifferentiated (stem) cells, are as yet not defined. Lacking such a defined antigen, a quick ELISA test could not be performed and an alternative test method had to be developed. We used ethanol fixed tissue sections, as described by Dinjens et al.[7] in order to approximate the native state of the tissue, and proteolytic enzyme digestion to find immunoreactivity on formalin fixed tissues.

Antibodies obtained

Simple immunization: At least thirty mice were immunized with cell homogenates either from cell lines or from human colon crypts without additional efforts to tailor the immunoreactivity. Most obtained hybridomas showed variably intense unselective staining of mucosal cells. Antibodies with a differential staining pattern are summarized in Table II. One of the developed antibodies, obtained after immunization with undifferentiated HT-29 cells, did show immunoreactivity at the bottom of the crypts of the small intestine. This antibody (IVF3) appeared to recognize a protein present in Paneth cells, probably lysozyme. Also two antibodies were developed after one intrasplenal injection of cells from the celline NCI-H716, recognizing respectively enterocytes all along the crypt and endothelial cells (monoclonal antibodies 10F9 and 4A3). Both appeared to be of IgMκ subtype. 10F9 reacted with formaline fixed tissues after pepsine digestion. This antibody is directed against a peptide epitope with a molecular weight of 54 kD. A last example of an antibody developed with this

Table II.

Procedure	antibody	isotype	pattern
HT-29 undifferentiated	IVF3	N.D.	paneth cells
NCI-H716	10F9	IgMκ	enterocytes
NCI-H716	4A3	IgMκ	endothelium
$CaCO_2$ undifferentiated	6B3F4	IgMκ	nuclei
Blocking of antigens	5E9	IgMκ	"immature" goblet cells
cyclophosphamide	2C8	N.D.	CEA

N.D. = not determined.

method (6B3F4),was obtained after one intrasplenal injection of undifferentiated $CaCO_2$ cells. 6B3F4 stains nuclei of all colonic crypt epithelial cells and is of IgMκ class.

Blocking of common crypt cell antigens: Using anti-mucin antisera as blocking agents, one interesting antibody was developed (5E9). This antibody shows immunoreactivity with goblet cells in the lower half of the crypts in the large intestine and is of IgMκ class. Biochemical characterization revealed that this antibody recognizes a carbohydrate (O-glycan) epitope on Muc2. An extensive description is given in chapter 3 of this thesis.

In vivo selection of the immune system: The cyclophosphamide elimination strategy in the end proved to be too toxic: of a total of 10 mice immunized with the cyclophosphamide protocol, 7 died during the treatment. Of the remaining three mice, sera yielded polyclonal antibodies with weak immunoreactivity after three treatments of in total 9 times a dosis of 100 mg/kg cyclophosphamide. Ideally, the cyclophosphamide treatment would have to be repeated untill the immune sera would no longer show immunoreactivity towards the antigen mixture, but in our hands not one single mouse did survive following this protocol. Therefore, we decided to limit the cyclophosphamide treatment to three cycles and followed with an injection of undifferentiated cells. One of the hybridomas we isolated showed immunoreactivity against carcinoembryonic antigen (CEA) (2C8).

Discussion

In order to obtain antibodies against antigens associated with undefined and less common antigens, such as undifferentiated cells, different approaches are avail-

able. The first and the most direct approach to produce monoclonal antibodies against rare antigens present as a small proportion of the total content of a protein mixture is popularly known as the shotgun approach[8,9]. In this most simple protocol, the mixture of proteins, including the protein which is the presumed target antigen is used to immunize the animal. The obtained hybridomas are screened often by reactivity patterns to cells or tissues. Here the chance of success is a question of numbers: the higher the number of hybridomas tested, the higher the chance to find what is desired. Two additional factors affect the success of the method. The first is the fraction of the target antigen within the total protein mixture. If the protein of interest only makes up a very small proportion of the mixture, the number of hybridomas to be tested would, statistically, have to be very large. The second is the immunogenicity of the desired protein in comparison with the other proteins in the mixture. If the protein of interest is weakly immunogenic compared to the other antigens in the mixture, a favourable outcome of the enterprise is rather unlikely. The shotgun approach did yield antibody producing clones, but none with the desired staining pattern of crypt stem cells. This might be due to -1- feeble presence of crypt stem cell specific antigens and -2- weakly immunogenic crypt stem cell antigens compared to the other antigens in the lysate. Given the very restricted number of crypt stem cells their low quantity might have been insufficient to elicit an immune response.

The second method involves the use of blocking antibodies. In this approach, an initial immunization will generate antibodies against all antigens in the lysate of differentiated cells[10]. Then, a lysate of the undifferentiated cells is incubated with this antiserum and the mixture is used to immunize a second series of mice. In theory, using this method, the production of desired immunoreactivities is favored because the initially generated antiserum will block the generation of immunoreactivities recognizing differentiated cells. The exact mechanism responsible for the presumed restriction of the immune response is not known, but Jerne's network hypothesis could explain how this might occur. The added antiserum induces synthesis of anti-idiotypic antibodies, which prevent generation of antibodies with immunoreactivities crossreacting with those in the initial antiserum. This method has as advantage that it allows a certain tailoring of the immune response contrary to the shotgun approach. It does require, however, the availibility of a set of closely homologous proteins of which one does while the other does not have the target antigen. This procedure resulted in an antibody recognizing goblet cells in the lower half of the crypt (5E9). Its properties will be described extensively in chapter 3 of this thesis.

As third method we used the cyclophosphamide elimination strategy[11]. This approach is based on the clonal expansion of B lymphocytes during the expansion

phase of a humoral immune response. These rapidly dividing cells can be preferentially killed using cyclophosphamide. A procedure for selective elimination of lymphocytes through the use of cyclophosphamide has been developed by Matthew and Sandrock[12]. Essentially, in this procedure mice are immunized intraperitoneally with the antigenic preparation containing a complex mixture of antigens but without the target antigen. After boosting, cyclophosphamide is administered and subsequently the antigen of interest will be injected. This procedure yielded one interesting immunoreactivity pattern. Upon characterization of this antibody it appeared to recognize CEA. We had to abandon this attractive strategy because it appeared to be too toxic: few of our mice survived.

As we had no defined antigen, immunization was done with cell lysates. In order to find an interesting hybridoma we had to test on tissue sections, as our stem cells are largely topographically defined. Ideally, in order to approximate a native state of the antigens, immunohistochemistry should be performed on fresh frozen colonic mucosa. However, because of the complexity of such an approach, we decided to use ethanol as a fixative and embed the tissue in paraffin. Tissue fixation as well as paraffin embedding reduce the immunoreactivity of an antigen[13,14]. Some antigens detectable in small quantity in fresh frozen tissue may be completely lost during routine processing and paraffin embedding. While no single fixative is ideal for all antigens, satisfactory results for many different antigens can be obtained following fixation in buffered formalin, provided that the exposure to this fixative is limited to a few hours[15,16]. Also denaturing but non-cross linking fixatives, as for example ethanol, can be used to retain antigens immunoreactivity. Ethanol better preserves proteins but tissues fixed in ethanol undergo marked shrinkage and the resulting morphology is poor[17].

In principle, the test substrate for hybridoma selection should be treated identical to the substrate on which the hybridoma will be used. As in pathology the test substrate mostly is formalin fixed paraffin embedded tissue it would have been consistent to use such material. We chose ethanol fixed tissue in order to expand the number of interesting hybridomas, on the assumption that cross-linking due to formalin fixation would be more destructive than ethanol fixation and immunoreactivity of formalin fixed epitopes might be retrieved using protease digestion or microwave heating of tissue sections.

Based upon our experience, which clearly is not a systematic evaluation of immunization protocols, we conclude that antibody mediated tailoring of immunization is a promising approach towards increasing the specificity of immunization with non defined antigens. In general, the choice of immunization protocols will be determined empirically and guided by the requirements of each specific experiment.

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Chapter 3

Expression of a marker for colonic crypt base cells is correlated with poor prognosis in human colorectal cancer

Anneke A.M. van der Wurff, Joop ten Kate, Patrick T.J. Marx, Edith P.M. van der Linden, Cor C.L. Beek, Floris-Jan Bovelander, Jan Dekker, Winand N.M. Dinjens, Maarten F. von Meyenfeldt, Jan-Willem Arends, and Fred T. Bosman.

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Introduction

In colorectal cancer, tumour stage as expressed in the Dukes classification[1] is still one of the best prognostic indicators. However, the behaviour of individual tumours within one stage is not uniform and therefore additional prognostic factors are needed for more accurate determination of tumour behaviour. One such prognostic indicator is histological grading[2] which is however subjective and of limited significance.

In search for independent prognostic indicators, in addition to proliferation, oncogene status and ploidy, differentiation markers have been developed for the cell lineages in the colonic epithelium, which include columnar cells, mucin producing goblet cells and neuroendocrine cells. In a large series of colorectal adenocarcinomas, markers for end stages of differentation did not seem to have prognostic value[3]. Only neuroendocrine markers correlated with worse prognosis[4,5,6] which might reflect the relative immaturity of these cells, which are located basally in the crypt.

All colonic epithelial cells are thought to arise from a common progenitor cell, the stem cell [7,8,9] which is located at the bottom of the crypt and gives rise to daughter cells with proliferative capacity. During movement upwards to the direction of the lumen, the proliferative ability of the committed precursor cells for each of the three cell lineages is lost and gradual differentiation takes place. The fully differentiated cells are eventually shed into the bowel lumen.

For normal large bowel mucosa the stem cell concept appears valid and has gained wide acceptance. For carcinomas several observations support this concept. Firstly, in a small proportion of colorectal carcinomas a wide spectrum of differentiation has been found[10,11]. These findings are strongly in favour of the origin of these tumours from a multipotential stem cell and these "stem cell" carcinomas seem to have a particularly unfavourable prognosis. Secondly, in colon cancer cell lines induction of differentiation reduces the proliferation index[12,13,14,15,16]. Thirdly, studies on human colon cancer cell lines have shown that these may exhibit a variable degree of proliferative activity, clonogenic potential and cell differentiation [7,18,19,20]. Finally, clonogenic assays generally show cloning efficiencies of tumour cell populations as low as 0,001% [21], suggesting that the proliferating tumour cell fraction in human tumours is small.

Our working hypothesis is that in analogy with normal colonic mucosa, in colorectal cancer tumour stem cells exist. The proliferative capacity and stage of maturation of the tumour stem cells would be an important determinant of tumour behaviour: the more immature the tumour stem cell, the higher the proliferation index and the less differentiated and the more aggressive the tumour. To test the validity of this concept, we set out to generate monoclonal antibod-

ies, which selectively stain crypt base cells, assuming that antigens in immature crypt base cells might also be expressed in colonic adenocarcinomas. In this paper, we describe the generation and characterization, by way of immunohistochemistry and immuno- and biochemical analysis, of a new monoclonal antibody, 5E9, which selectively stains goblet cells in the lower half of the colonic crypt. The prognostic significance of its immunoreactivity was tested on a large series of colorectal adenocarcinomas. High expression of the 5E9 epitope appeared to be correlated with a tendency for poor prognosis in Dukes stage B3 patients.

Materials and methods

Monoclonal antibody production. As a source of colon crypt epithelial cells, we used mucosa from neoplastic as well as non-neoplastic colectomy specimens. In case of specimens resected because of cancer, mucosa at least at five cm distance of the tumour was used. This was incubated, after decontamination with 0.04% (m/v) Sodium hypochlorite in PBS and 0.5 mmol/L DTT in PBS to free the sample of mucus, with 0.05 mmol/L EDTA in calcium and magnesium-free Hanks balanced salt solution (CMF-HBSS, Gibco, Paisley, Scotland) in a spinner flask (Gibco) according to Ten Kate et al.[22]. The mucosa with the remaining crypt bottom parts was incubated according to Whitehead et al.[23] in a sterile petridish containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA, Merck, Darmstadt, Germany), 1 mmol/L ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA, Sigma Chemical Company, St. Louis, MO) and 0.5 mmol/L dithiotreitol (DTT, Sigma) in phosphate buffered saline (PBS, pH=7.4), 30 min at room temperature. The crypts were liberated by vigorous shaking and harvested by centrifugation. The cells were lysed in buffer containing 5 mmol/L $NAHPO_4$, 1 mmol/L EDTA, 1 mmol/L β-mercaptoethanol, 0.125% (m/m) deoxycholate, 0.125% (v/v) Triton X-100, 250 mmol/L sucrose (pH = 6.4) mixed with protease inhibitors (1 mmol/L phenylmethylsulphonyl fluoride (Sigma), 2 mmol/L N-ethylmaleimide (Sigma)). To reduce the availability of major mucin antigens[24], the lysate containing the crypt bottom epithelium was incubated with antisera against colonic epithelium generated in mice and mixed with monoclonal antibodies against colonic mucins (Parlam 9 and 10)[25]. Bound antibodies were precipitated with Sepharose protein A (Pharmacia, Uppsala, Sweden)(1 hr 4°C). Balb/c mice were immunized intrasplenally with 200 µL of the final lysate, 72 hr prior to fusion. As repeated intrasplenal immunizations appeared not to be feasible, due to a high chance of rupture of the spleen, the mice were immunized only once. Spleen cells were fused with Sp 2-0 myeloma cells and selected using standard procedures[26]. Supernatant was tested

on sections of normal colon and small bowel tissue specimens fixed in alcohol 70% as described by Dinjens et al.[27] to avoid epitope destruction by aldehyde fixation. Antibody producing wells were cloned and recloned by limiting dilution untill all clones were productive. Isotyping was performed by immunohistochemistry on colonic epithelium using isotype specific secondary antibodies (Mouse typer, Biorad, Richmond). Specific positive and negative controls for each of the heavy and light chains were used.

Immunoreactivity. Normal adult human tissues and tumours were retrieved from our files. Of each tissue, at least two specimens were tested and all tissues were obtained fresh, fixed in 4% (v/v) buffered formalin, dehydrated and embedded in paraffin. Also, frozen tissue specimens were used. Sections of 4 µm thickness were cut and stained with an indirect immunoperoxidase technique. For antigen retrieval, sections were digested with proteases (15 min 37°C) in concentrations ranging from 0.4% to 0.025% (m/v) for trypsin (in 0.4% $CaCl_2$, pH=7.8) and pepsin (0.1 mol/L HCl). Also, pronase in 0.05 mol/L Tris HCl (pH=8) was tested in concentrations of 0.04% to 0.0025% (m/v) (30 min 37°C). Immunoperoxidase staining was performed using rabbit anti-mouse IgG conjugated to horseradish peroxidase as detection system and diaminobenzidine(DAB)-H_2O, as chromogenic substrate. For post-embedding immunoelectron microscopy, 1 mm^3 fragments of human colon mucosa taken from freshly obtained surgical specimens were fixed in 0.1 mol/L phosphate buffer (pH = 7.2) containing 1 % (m/v) acroleine and 0.4% (m/v) glutaraldehyde at 4°C for 4 hours. Tissues were transferred and stored in a sucrose buffer of 1 mol/L sucrose in 0.1 mol/L phosphate buffer (pH = 7.2) with 1% (v/v) paraformaldehyde at 4°C until further processing for standard Lowicryl embedding[28]. After impregnation of the tissues with Lowicryl K4M (Baltzers, Liechtenstein) polymerization was performed under UV exposure at -35°C. Ultrathin sections were first incubated with 10% (v/v) normal goat serum (NGS, Dakopatts, Glostrup, Denmark) in PBS (30 min at room temperature), second with the primary antibodies (1 h at room temperature) and finally with 10 nm colloidal gold-labeled goat-anti-mouse antiserum (GAM-10, Aureon, Wageningen, The Netherlands) under microwave irradiation at 150 W for 30 min[29]. The immunogold-silver enhancement was done by magnification of the gold particles by precipitation of metallic silver (Aureon R Gent Developer and Enhancer, applied 2 min at room temperature in the dark; Aureon). After rinsing in distilled water the sections were dried. The sections were contrasted with uranyl acetate and lead citrate. Micrographs were taken on a Zeiss EM 902 at 80 kV. Control sections were incubated with PBS. Background staining was always negligible.

Biochemical characterization. A polyclonal antiserum was raised against human colonic mucin as described previously (anti-HCM)[30]. The characterization of

the mucin epitopes recognized by 5E9 was performed by Western- and spot-blotting of purified colonic mucin, as described earlier for rat gastric mucin[31]. Briefly, purified mucins were either run on 4% SDS-PAGE and blotted onto nitrocellulose or purified mucins were spotted directly on nitrocellulose using a vacuum-operated spot-blot apparatus (Biorad, Richmond, USA). Blots were incubated either with anti-HCM (for reasons of comparison) or spent 5E9 hybridoma culture medium, diluted 1: 100, followed by incubation with goat-anti-rabbit IgG conjugated to HRP and stained with DAB. Specificity for peptide and carbohydrate epitopes was tested separately as follows:

1. Proteinase K sensitivity prior to spot-blotting (to determine the possible peptide nature of the epitope).
2. Epitope sensitivity towards DTT reduction (to determine whether or not 5E9 detects a conformational peptide epitope).
3. Oxidative destruction of carbohydrate epitopes by periodic acid (to determine the carbohydrate nature).
4. Incubation of 5E9 with the protease-resistent glycopeptide of HCM (as an alternative method to show reactivity towards carbohydrate-epitopes). To this end, HCM was digested 24 h with 10 µg/mL proteinase K (Merck) in 50 mM Tris, pH 7.4, at 37°C. The proteinase K was inactivated by addition of an excess of phenylmethylsulfonyl fluoride (Sigma). The proteinase-resistent glycopeptide was subsequently recovered in the void volume after Sephacryl S-200 (Pharmacia) gel filtration in 50 mM Tris, pH 7.5. Isolated HCM-glycopeptide was incubated for 1 h at room temperature with the antibody prior to addition to the spot-blotted mucin.

The recognition of mucin-antigens was further tested in metabolic labeling experiments of colonic biopsies followed by immunoprecipitation of radiolabeled mucins, as was described in detail elsewhere[30].

To test whether the epitope recognized by 5E9 is a sialylated carbohydrate chain, neuraminidase digestion was performed according to Jass et al.[32]. 1 mg neuraminidase (Clostridium perfringens, Fluka 72202, Lot 326138/1) was dissolved in 0.425 ml of 0.05 M Tris-HCL buffer, pH 7.0, containing 0.01% (w/v) $CaCl_2$. Saponification was achieved by immersing the slides in 0.5% KOH in 70% (v/v) alcohol (30 min at room temperature) Thereafter the slides were rinsed in 70% (v/v) alcohol and washed in tap water for 10 min. As a positive control the lectin PNA (Boehringer Mannheim) was used with and without saponification.

Statistical analysis. Colorectal adenocarcinomas of 350 patients were entered in two multicenter prospective clinical trials between 1979 and 1981. One trial was designed to compare patient survival after treatment of colonic cancer by conventional surgery or the no-touch isolation technique[33]. The other trial was

conducted to compare survival in rectal cancer patients with or without pre-operative radiotherapy. These patient series did not differ in overall survival and therefore, for the purpose of the present study, the patient groups were pooled. During the first three years follow up took place every three months and every six months between three and five years after initial diagnosis and surgery. Standard protocols were conducted with routine blood counts and chemistry studies (including CEA levels) at each visit and liver ultrasound, chest X-ray and colonoscopy annually, to evaluate recurrence of disease and disease related death. After the initial five year follow-up, during subsequent years only the time and cause of death were registered. In this study, failure was defined as death due to recurrent disease, excluding post-operative mortality within thirty days and non-related death. The correlation between monoclonal antibody 5E9 expression and other colorectal differentiation markers, Dukes stage, differentiation grade, tumour size, localisation of the tumour within the large intestine, presence of (lymph-) angioinvasion or perineural invasion, lymphnode status and central lymphnode involvement was investigated in two way frequency tables using BMDP PC90 Statistical Software, program 4F (BMDP Statistical Software, Inc., Los Angeles, CA). Interdependency of 5E9 expression and these variables was tested with Pearsons χ^2 method. Scoring was done by two independent pathologists. 5E9 expression was compared with markers for the colorectal epithelial cell lineages. Mucin production was detected with the monoclonal antibody Parlam 3[25], which detects a glycoprotein present in normal goblet cells and with the High-Iron-Diamine Alcian Blue (HID-AB) technique, which stains sulphated mucins brown. Columnar cells were identified by immunoreactivity for secretory component (SC). Neuroendocrine differentiation was detected with a monoclonal antibody against chromogranin A, the major component of neurosecretory granules.

As a clinicopathological staging system we used the Dukes classification[1]. Differentiation grade was assessed according to criteria as outlined by Blenkinsopp[2]. The prognostic value of 5E9 expression with respect to survival was investigated by univariate analysis, using Kaplan-Meier estimations and the stratified logrank test, with the program 4F, BMDP PC90 Statistical Software. To exclude the possibility of a type I error in the statistical analysis, in addition a Bonferroni test was performed.

Results

Immunohistochemistry of the monoclonal antibody 5E9. With the described immunization protocol, the IgMκ monoclonal antibody 5E9 was obtained, staining goblet cells located at the crypt base of normal colorectal epithelium (Fig.

1a). For immunohistochemical experiments at light microscopical level, immunostaining on standard paraffin embedded tissue with spent hybridoma supernatant was optimal in a dilution of 1:50 in PBS-1% bovine serum albumin (BSA), after enzyme digestion of the tissue sections with 0.01% pepsin in 0.1 mol/L HCl (15 min 37°C). Comparison of unfixed cryostat sections with paraffin sections after pepsin treatment showed an identical staining pattern for both conditions. The immunoreactivity pattern of the monoclonal antibody 5E9 in normal and malignant human tissues is shown in Figures 1 and 2. Normal mucosa from neoplastic as well as non-neoplastic colectomy specimens (proximal and distal colon) was used. To exclude the possibility that 5E9 recognizes a major blood group antigen we stained colon mucosa samples from patients with different ABO blood groups. In all groups an identical staining pattern was obtained. In the digestive tract, immunoreactivity was noted in submucosal glands of the esophagus, duct epithelium of the parotid gland, goblet cells at the lower half of the crypt in the duodenum, some pancreatic duct cells (Fig. 1b) and in the mucosa lining the gallbladder (Fig. 1c). Pancreatic islets were not stained. In normal colon mucosa, from neoplastic as well as non-neoplastic resection specimens, only crypt base cells showed 5E9 immunoreactivity. Strikingly, immunoreactivity was observed in all goblet cells in colonic epithelium adjacent to adenocarcinomas (transitional mucosa. Fig. 1d). In the stomach, reactivity was noted only in some goblet cells in areas of intestinal metaplasia (Fig. 1e). In the urogenital tract, staining was observed in endometrial and endocervical glandular epithelium (Fig. 1f). Furthermore, reactivity was found in some acinar and duct cells of the breast. Also in prostate and bladder epithelium focal reactivity was observed. In normal ovary tissue, no immunoreactivity was found. In cystadenomas and adenocarcinomas of the ovary, however, whether serous or mucinous, focal immunoreactivity was noted. In the respiratory tract, bronchial epithelium, pneumocytes type II and few goblet cells in the lining tracheal epithelium showed staining. Carcinomas in urogenital and respiratory tract showed mostly a focal immunoreactivity pattern.

Colorectal adenocarcinomas showed 5E9 immunoreactivity in 33% of our 297 cases. The proportion of positive cases did not differ appreciably for different periods of collection of patient material, which makes it unlikely that variations in tissue preparation techniques or limited antigen stability might have influenced the immunohistochemical results. The staining was often intracellular, but also apical membrane associated; often some single cells were homogenously stained, but occasionally diffuse staining was observed (Fig. 2). By immunoelectron microscopy the reactivity was located intracellular on the outer rim of mucin vacuoles (Fig. 3). Staining of the Golgi complex was not observed.

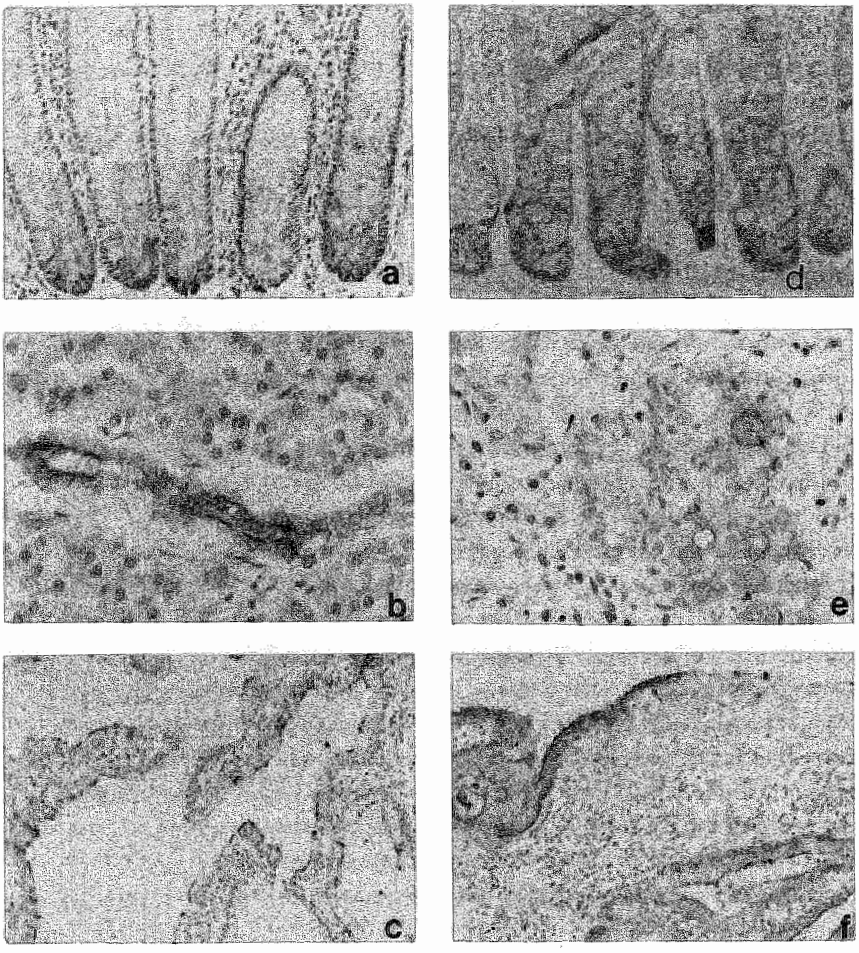

Figure 1. Immunoreactivity pattern of the monoclonal antibody 5E9 on various normal human tissues. a: normal colonic muscosa; b: pancreas; c: gallbladder; d: transitional colonic mucosa; e: intestinal metaplasia in the stomach; f: cervix uteri: squamocolumnar junction.

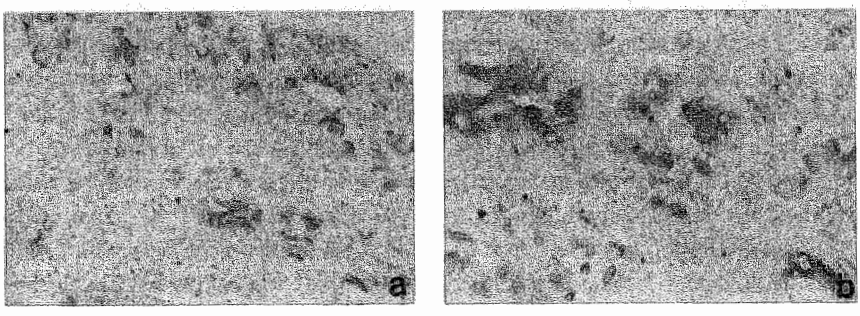

Figure 2. 5E9 immunoreactivity pattern in colorectal adenocarcinoma. a: overview; b: detail

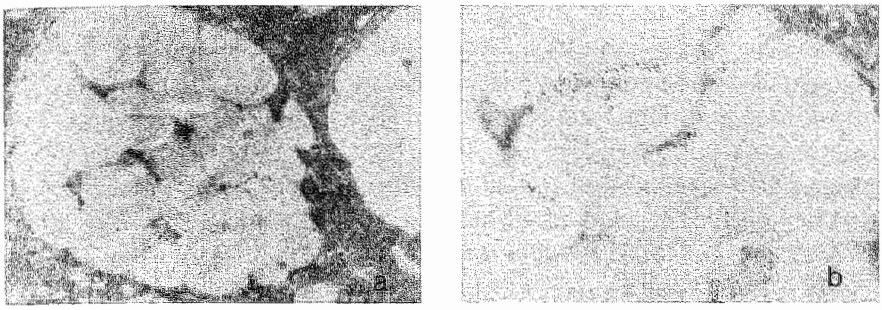

Figure 3. 5E9 immunoelectron microscopy. 5E9 immunogold reactivity is located intracellular in goblet cells on the outer rim of mucin vacuoles. a: overview; b: detail

Biochemical characterization of the 5E9 epitope. As colonic mucosa was used as antigen source, mucins seemed to be the likely candidates for the antigen recognized by 5E9. Therefore, studies were undertaken to define this antigen, using purified HCM for comparison. Previous studies had shown that anti-HCM specifically recognizes polypeptide-epitopes of Muc2[30]. On Western blotting, HCM was recognized by both anti-HCM and 5E9 as a 550 kDa band. This band was previously identified as human colonic Muc2, the major secretory mucin in the colon, which is produced in the colonic goblet cells[30]. Digestion with proteinase K resulted in a decrease of the immunoreactivity of 5E9 towards HCM, while reduction by DDT had no effect. Oxidation of carbohydrate-epitopes by periodic acid resulted in complete loss of reactivity. Also the pre-incubation of 5E9 with the HCM-glycopeptide, isolated after extensive proteinase K-digestion, inhibited the binding of 5E9 with spot-blotted HCM. The reactivity of anti-HCM towards HCM, which reacts primarily with peptide epitopes, showed a converse reactivity compared to 5E9: proteinase K destroyed all reactivity, while oxidation by periodic acid and pre-incubation with HCM-glycopeptide had no effect on reactivity. These results indicate that 5E9 recognizes most likely a carbohydrate-epitope of HCM. In metabolic labeling studies, using 5E9, we were unable to immunoprecipitate [^{35}S]sulphate-labeled mature Muc2, or its [^{35}S]amino acids-labeled precursor. As a positive control, immunoprecipitations from the same tissue homogenates, using anti-HCM, yielded the expected 600 kDa [^{35}S]aminoacid-labeled Muc2-precursor, and the 550 kDa [^{35}S]sulphate-labeled mature Muc2.

5E9 immunoreactivity could not be abolished by neuraminidase digestion with or without prior saponification. Lectin PNA, used as a positive control, showed the expected reactivity pattern.

Clinicopathological analysis. The 350 patients in this study were 165 men and 185 women, ranging in age from 29 to 91 years (the median age was 69 years). 5E9 staining was performed on available tissue specimens of 297 patients (192 colonic adenocarcinomas and 105 rectal carcinomas). These were comparable in Dukes stage and histological grading to the overall population. To obtain subgroups suitable for statistical analysis, the subgroups were combined: group A tumours consisted of colorectal adenocarcinomas of Dukes A, B1 and B2, group B consisted of the Dukes B3 while group C combined Dukes C1 and C2 tumours. Group D contained the Dukes D. Of the tumours, using the above mentioned Dukes combinations, 63 were group A, 117 group B, 91 group C and 26 group D. The number of well differentiated tumours was 31, 227 tumours were moderately differentiated and 32 poorly differentiated (7 tumours were not graded). 5E9 positive cells were found in 97 tissue specimens of colorectal cancer. Multiple two way frequency tables were prepared yielding two positive results, namely with Chromogranin A and with HID-AB expression. There seems to be a trend towards a positive correlation of 5E9 expression with neuroendocrine differentiation (p=0.01) as well as with HID-AB, a sulfomucin stain (p=0.04). Kaplan Meier survival analysis for the patient subgroups is illustrated in Fig. 4. A stratified log rank test was used, which showed non-significance (p=0.265). If however the different Dukes groups are taken into account, 5E9 immunoreactivity was noted in 23, 39, 31 and 11 cases in respectively group A, B, C and D. It appeared that in group B (Dukes B3) 5E9 expression seems to be positively correlated with worse prognosis (p=0.037). In the Bonferroni test, to exclude a type I error, this result was not sustained.

Discussion

The histological classification of colorectal adenocarcinoma has not changed in recent years. In addition to adenocarcinomas, mucinous, signet ring and undifferentiated carcinomas are distinguished (WHO). Furthermore, grading is performed in a three grade system[2], which falls short in reproducibility and has limited prognostic significance. Various attempts have been made to improve classification using markers for enddifferentiational stages of the cell lineages in colonic epithelium[3,4,5,6]. Secretory component (SC) is a marker for enterocytes, lysozyme for Paneth cells, different mucin markers identify goblet cells and neuroendocrine markers neuroendocrine cells. Mucins, SC and lysozyme are often co-expressed in a large proportion of the adenocarcinomas and for these markers a consistent prognostic significance has not been reported. With regard to neuroendocrine markers, expression of Chromogranin A was repeatedly found to correlate significantly with worse prognosis[5,6].

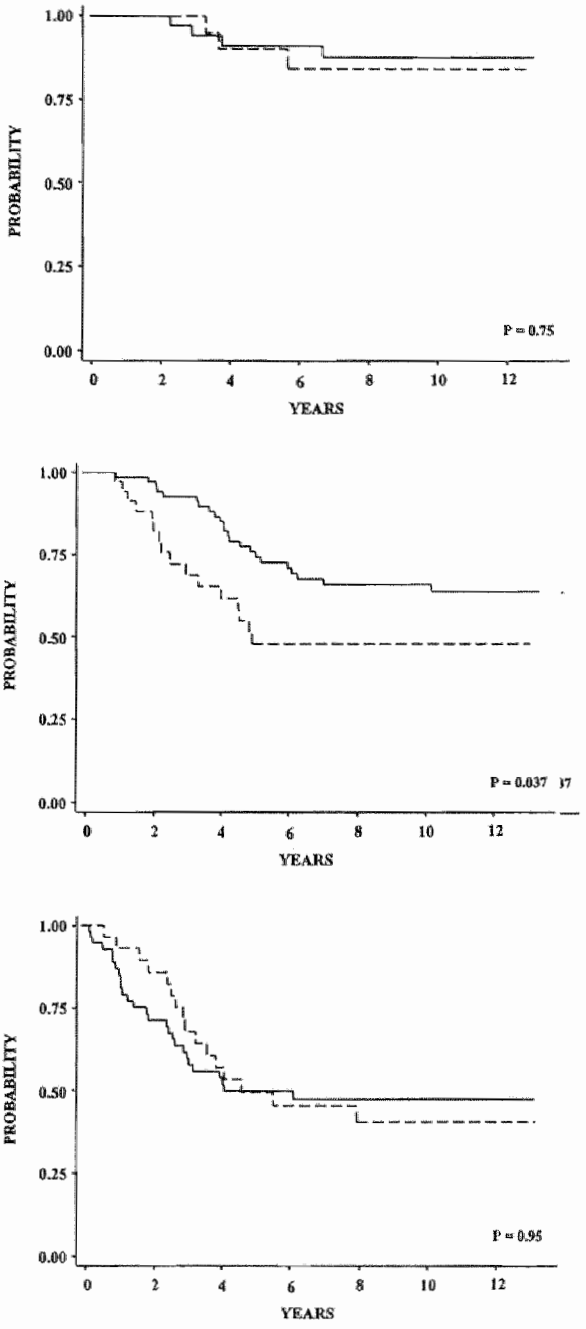

Figure 4. Kaplan Meier survival curves for colorectal adenocarcinoma with 5E9 immunoreactivity. a: Dukes A, B1 and B2. p=0.75. (N=63); b: Dukes B3. p=0.037. (N=117); c: Dukes C1and C2. p=0.95. (N=91); dotted lines: 5E9 expression; solid lines: No 5E9 expression

In looking for new markers for classification of colorectal cancer we reasoned that the behaviour of the tumour is largely determined by the proliferating stem cell fraction and not by terminally differentiated cells. Therefore, it would be plausible to look for markers for the undifferentiated (stem) cell fraction of the tumour. In search for such markers we reasoned that crypt base cell associated antigens might also identify immature tumour (stem) cells.

Using as an antigen source epithelium of crypt bases, which were incubated with antibodies against major mucin epitopes and with antisera against colonic epithelium generated in mice to block for dominant epitopes, we succeeded in developing an antibody against a subset of goblet cells located in the lower crypt half, around and just above the proliferation zone. The epitope of this monoclonal antibody is expressed in a diversity of human tissues, normal and malignant, but shows a very specific staining pattern in the colon. Immunoelectron microscopy showed reactivity in the outer rim of mucin vacuoles in goblet cells. It is of interest that in the digestive tract 5E9 staining was found in goblet cells in intestinal metaplasia in gastric mucosa as well as in Barrett's esophagus. Both conditions carry an increased risk for the development of adenocarcinoma. In contrast to staining of basally located goblet cells in normal colonic mucosa, all goblet cells were stained in transitional mucosa neighboring adenocarcinoma. Ultrastructural studies have indicated that crypt epithelial cells in transitional mucosa show slower maturation than normal[34]. The increase of 5E9 positive cells in transitional mucosa could be the result of such a delay in the process of crypt cell maturation and this observation would be in keeping with the lower differentiation level of 5E9 expressing goblet cells.

In search for the epitope recognized by 5E9, immunoblotting showed that 5E9 recognizes an epitope present on an HCM associated glycoprotein, identified as human colonic Muc2[30]. The blocking of 5E9 staining by periodic acid oxidation and by preincubation with the HCM-glycopeptide indicates that 5E9 recognizes a carbohydrate (O-glycans) epitope on Muc2. Although the monoclonal antibody recognized Muc2 in blots, it was not able to retrieve the antigen by immunoprecipitation from homogenates. Possible explanations could be scarcity of the O-glycan epitope, the absence of an O-glycan in Muc2[35] or low affinity of 5E9, which is not entirely unlikely given the IgM isotype of the antibody. 5E9 stained specifically the mucin granules in goblet cells low in the colonic and small intestinal crypts of all individuals tested, indicating that the carbohydrate epitope was not a blood-group antigen nor an antigen covered by blood-group saccharides. The crypt base specificity of 5E9 staining suggests that the Muc2 epitope, recognized by 5E9, becomes masked when the goblet cells differentiate further during their migration upward from the bottom of the crypt. This could imply that the O-glycosylation-pattern of Muc2 changes when the crypt cells

migrate upward. The epitope recognized by 5E9 seems not to be sialic acid given the insensitivity to neuraminidase treatment with or without prior saponification. In the literature other monoclonal antibodies like SIMA[36] and STn[32] with a comparable immunoreactivity pattern are described. These antibodies recognize sialylated carbohydrate chains.

To evaluate the potential prognostic significance of 5E9 expression in colorectal adenocarcinomas, we stained a series of human colorectal carcinomas and compared the 5E9 status with various earlier studied pathological variables. We noted a trend towards 5E9 expression along with Chromogranin A defined neuroendocrine and HID-AB defined sulphomucin expression. Both characteristics were earlier found to be associated with worse prognosis[5,6]. The stratified log-rank test did not yield any significance. In our group A (Dukes A1, B1 and B2) and group C carcinomas (Dukes C1 and C2), 5E9 staining did not have any prognostic significance. In Dukes B3 patients however, a group of patients with a heterogeneous chance of survival, 5E9 appeared to be a positive prognostic variable, identifying a subgroup of patients with a worse prognosis. This enhanced expression of the 5E9 epitope in prognostically unfavourable Dukes B3 adenocarcinomas can indicate that a change in the O-glycosylation of Muc2 in colonic tumour cells might take place during progression towards recurrent (incurable) disease.

In conclusion, we have developed a new monoclonal antibody which most likely recognizes a non-terminal carbohydrate epitope on the Muc2 O-glycans in the colon and which reacts specifically with crypt base cells in colonic mucosa. In group B, Dukes B3 adenocarcinomas, 5E9 staining identifies a subset of tumours with a tendency for worse prognosis. Studies on more cases are needed to show whether these results prove to be statistically significant. Also the clinical value of 5E9 for colorectal cancer classification needs to be further evaluated.

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Chapter 4

L-CAM expression in normal, premalignant and malignant colon mucosa

Anna A.M. v.d. Wurff, Joop ten Kate, Edith P.M. v.d. Linden, Winand N.M. Dinjens, Jan-Willem Arends, Fred T. Bosman.

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Introduction

The cell adhesion molecule L-CAM, also known as E-Cadherin, Uvomorulin, Arc-1 or cell CAM 120-80, plays an important role in cell-cell interactions. Damsky et al.[1] were the first to identify L-CAM in humans. L-CAM plays an essential role in embryonic development and morphogenesis, as well as in the maintenance of the normal structure and function of adult tissues by way of calcium dependent homophilic adhesion between epithelial cells[2].

Shimoyama et al.[3] and Eidelman et al.[4] already reported that L-CAM expression can be found in all normal human adult epithelia. Shimoyama et al.[3] described L-CAM reactivity at the intercellular border only, while Eidelman et al.[4] found a uniform expression of L-CAM along the entire cellular circumference in squamous epithelia of the skin, but in intestinal epithelium expression only along the zonula adherens. L-CAM reactivity was not found at the basal plane of the cell membrane facing the basement membrane. Studies on L-CAM expression in cancer have mainly focused on tumour cell lines. These studies have shown that transient down regulation induces invasive behaviour of tumour cells in parallel with loss of junctional complex formation. Vleminckx et al.[5] described how epithelial tumour cells, highly invasive in the embryonic chicken heart assay, lost their invasive potential after transfection with a cDNA encoding for L-CAM, and also did not invade into type I collagen matrices. These data indicate that L-CAM expression and invasive behaviour are inversely correlated. Therefore, L-CAM could be a marker in histopathology to discriminate between carcinomas with metastatic and those without metastatic potential.

Earlier reports[3,4,6,7] described L-CAM expression in a series of human tumours. These indicate that all adenomas and well differentiated adenocarcinomas express L-CAM in a pattern comparable with that in the corresponding normal tissues. In contrast, poorly differentiated carcinomas showed a weaker and heterogeneous expression pattern with L-CAM evenly distributed along the entire cell circumference. Shimoyama and Hirohashi[7] reported L-CAM reactivity on the cell surface in gastric carcinomas of the scattered type i.e. those cancer cells that did not show tight intercellular adhesion.

We hypothesized that in view of the possible role of L-CAM as an invasion suppressor, L-CAM immunoreactivity would be detected in colonic adenomas, but in not carcinomas.

The present study was performed to test this hypothesis by analyzing L-CAM expression in tissue specimens of normal mucosa, adenomas and carcinomas of the colon.

Materials and methods

Surgical specimens of 23 colorectal polyps (17 tubular and 3 tubulovillous adenomas and 3 hyperplastic polyps), 38 adenocarcinomas (16 Dukes A/B and 22 Dukes C) were obtained. Normal mucosa was excised from 20 colectomy specimens at a site at least 5 cm from the neoplasm. Tissues were snap frozen in isopentane and stored at -70°C until sectioning. Sections of 4µm thickness were cut, air dried, fixed in methanol for 1 minute at -20°C, dipped in acetone at -20°C and stained. To reduce non specific staining, endogeneous peroxidase was inactivated (0,5% H_2O_2 in methanol). An indirect immunoperoxidase method was used. Briefly, the sections were incubated with a monoclonal anti L-CAM antibody (6F9, Eurodiagnostics, the Netherlands) in a dilution of 1:80 in PBS-1% BSA (1 h. RT) and after three washings in PBS incubated with a rabbit anti mouse Ig-HRP conjugate (Dakopatts Denmark)(1 h. RT). After repeated washing in PBS, HRP activity was visualized with diaminobenzidine (DAB) and H_2O_2. Finally the sections were counterstained with haematoxylin and mounted with entellan. In control slides PBS-1% BSA was used without the monoclonal antibody.

For photography representative sections were stained using an immunofluoresence method. Sections were incubated with L-CAM as described above, but after three washings in PBS they were incubated with fluorescein isothiocyanate (FITC) conjugated rabbit anti mouse Fab fragments (Dakopatts Denmark) diluted 1:50 (1 h. RT). They were rinsed in PBS, mounted in Immunomount (Shandon Pittsburgh) and examined with a Leitz Dialux 20 microscope equipped for UV-epi-illumination.

Scoring was performed by two independent observers. The following nomenclature was used: (1) Normal i.e. the same pattern of L-CAM immunoreactivity as seen in normal colon mucosa: uniform expression along the intercellular borders. (2) Apical i.e. intensified immunoreactivity at the luminal cell surface and (3) negative: no immunoreactivity at all.

To differentiate between extractable and non-extractable L-CAM, we used a protocol based on the observation[8] that some L-CAM molecules might not be functionally linked with the cytoskeleton and therefore can easily be solubilized with Triton X-100. To this end, tissue sections were preincubated before and after fixation procedures as described, for different time periods (1 sec, 1 min, 5 min, 15 min, 30 min) with various concentrations of Triton X-100 (0,05%, 0,1%, 1%). As a control (for reduction of non specific background) tissue sections were preincubated with 0,5 M NaCl instead of Triton X-100. L-CAM staining was subsequently performed as described.

Results

Normal colonic epithelium. All normal mucosa specimens showed distinct non-polarized evenly distributed expression of L-CAM. Immunoreactivity was found along intercellular borders and not at the basal plane of the cell facing the basement membrane. (Fig. 1)

Colon polyps. The findings are summarized in Table I.

All 3 hyperplastic polyps studied displayed a staining pattern identical to that seen in normal mucosa. The 20 tubular and tubulovillous adenomas all demonstrated a normal staining pattern, four of which with intensified L-CAM expression at the apical cell surface. However, the staining was less than in normal mucosa.

Dukes A/B carcinomas. 15 out of 16 Dukes A/B carcinomas showed normal though weak L-CAM expression, six with apical L-CAM immunoreactivity (Fig. 2). One poorly differentiated carcinoma appeared to be negative.

Dukes C carcinomas. 19 out of 22 Dukes C carcinomas showed a normal though weak L-CAM expression, including eight carcinomas (six moderately differentiated and two poorly differentiated) with L-CAM immunoreactivity at the apical cell surface.

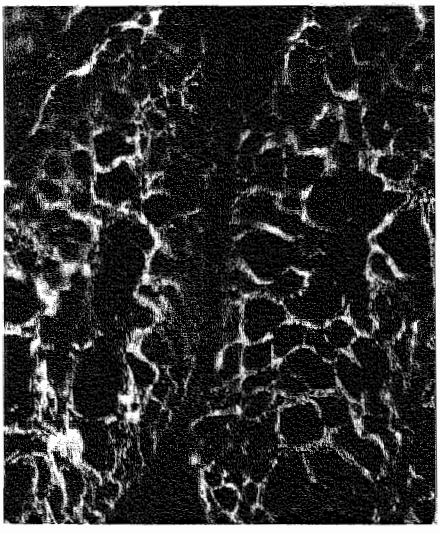

Figure 1. Normal L-CAM staining pattern in normal colon mucosa using an indirect immunofluorescence method with monoclonal anti-L-CAM antibody 6F9.

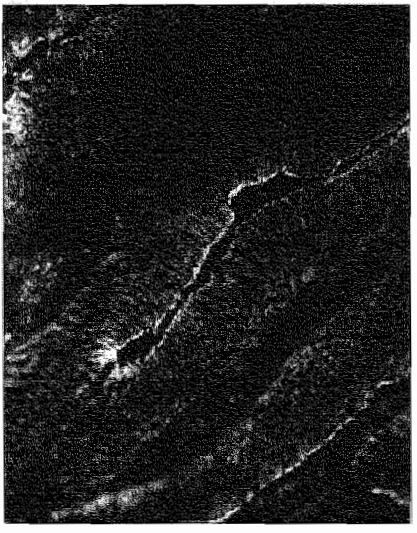

Figure 2. Apical L-CAM staining pattern in colon neoplasm using an indirect immunofluorescence method with monoclonal anti-L-CAM antibody 6F9.

Table I.

Adenomas

		L-CAM expression			
		Normal	Apical	Negative	Total
Differentiation	Well*	10	0	0	10
	Moderately well	8	3	0	11
	Poorly	1	1	0	2
	Total	19	4	0	23

* including three hyperplastic polyps

Dukes A/B

		L-CAM expression			
		Normal	Apical	Negative	Total
Differentiation	Well	3	0	0	3
	Moderately well	5	5	0	10
	Poorly	1	1	1	3
	Total	9	6	1	16

Dukes C

		L-CAM expression			
		Normal	Apical	Negative	Total
Differentiation	Well	4	0	0	4
	Moderately well	6	6	0	12
	Poorly	1	2	3	6
	Tota	11	8	3	22

In three poorly differentiated carcinomas no L-CAM expression was found.
In none of the neoplastic tissue specimens any difference in L-CAM reactivity according to area within the tumour (centre or periphery) could be found.
L-CAM expression after Triton X-100 pretreatment. All three colon tumours with apical staining pattern showed strongly reduced or negative L-CAM immunoreactivity after preincubation (1 sec) with 0,05% Triton X-100. The two tumour specimens with a normal staining pattern i.e. along intercellular borders, did not loose their L-CAM reactivity after Triton X-100 preincubation. Longer preincubation with higher concentrations Triton X-100 resulted in loss of morphology which made scoring impossible.
Control preincubation with 0,5 M NaCl or preincubation with Triton X-100 after fixation of the sections did not alter the apical nor the normal immunoreactivity.

Discussion

In the process of invasive growth, in which one of the critical steps is the detachment of epithelial junctions, L-CAM may play an important role. In studies on tumour cell lines[9,10], a very clear correlation between L-CAM expression and invasive behaviour is described. On the one hand, cells expressing L-CAM do not infiltrate into collagen matrices or chicken heart fragments. After incubation with monoclonal antibodies against L-CAM, however, they show invasive behaviour[11]. On the other hand, cells that do not express L-CAM have been shown to display invasive behaviour, but after transfection with a cDNA encoding for L-CAM[5], they convert into non invasive cells. It appears, therefore, that L-CAM functions as an invasion suppressor. These studies indicate that L-CAM expression must be down regulated once a cell becomes invasive.
It is interesting to note that a recessive oncogene, the DCC gene[12], shows homology with a member of the CAM gene family, namely N-CAM. This DCC gene is deleted in more than 70% of colon carcinomas. The deletion was regarded as a relatively late event during tumour progression. This does not parallel our observations in relation to the early down regulation of L-CAM.
Eidelman et al.[4] describe L-CAM immunoreactivity in more than sixty human normal tissues and tumours, including nine adenomas and five carcinomas of the colon. They concluded that normal colon epithelium shows polarized L-CAM expression and that in tumours L-CAM expression is less polarized, weaker and more heterogeneous. Other authors[3,6,7] concluded that L-CAM reactivity in carcinomas is less intense and more heterogeneous than in normal tissue. Shimoyama and Hirohashi[7] reported that 19 out of 54 gastric carcinomas expressed L-CAM on the surface of the cancer cells of scattered type carcinomas

and they postulated that defective expression of intrinsic factors, which anchor L-CAM to the cytoskeleton, are resposible for the aberrant expression of L-CAM, which by immunoblotting had a normal size.

Our studies confirm that in normal colon epithelium L-CAM is evenly distributed along cell-cell borders. A periluminal accentuation of reactivity (polarization) on the lateral sites of the apical cell pole, as described by Eidelman et al.[4], was also observed, but not in normal tissue. Confirming the observations of other workers[3,6,7], we found weaker and more heterogeneous immunoreactivity in carcinomas, without differences between metastasizing and non metastasizing carcinomas. L-CAM down regulation appeared to be an early and a gradual process in colon carcinogenesis, because it occurred in adenomas and was most striking in high grade carcinomas, where L-CAM expression was absent.

We found that a number of adenomas and carcinomas showed in addition to a normal though weaker staining pattern also staining at the apical cell borders. This staining pattern could be abolished by preincubation in Triton X-100 but not by preincubation with 0,5 M NaCl. Following the suggestion by Nelson et al.[8] we hypothesize that apical L-CAM is not linked to the cytoskeleton and therefore is more easily extractable with a detergent. This is in concordance with the suggestions made by Shimoyama and Hirohashi[7] concerning L-CAM expression in scattered type gastric carcinomas.

Although four poorly differentiated carcinomas were totally devoid of L-CAM reactivity, we think that the number of cases we studied is too small to draw any conclusions regarding L-CAM down regulation and differentiation. Shimoyama and Hirohashi[13] reported one aggressive hepatocellular carcinoma which was L-CAM negative.

In this study we demonstrated that L-CAM down regulation occurs in all colon adenomas and carcinomas, irrespective of stage of progression or localisation within the tumour, although there is a trend towards decreasing L-CAM expression along with decreasing grade of differentiation. A distinction between invasive and non invasive tumours cannot be made on the basis of L-CAM expression.

Studies at the single cell level might indicate that L-CAM down regulation is a temporary event, occurring only at the moment of invasive activity. Studies are now in progress to determine the validity of this concept.

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Chapter 5

L-CAM expression in lymph node and liver metastases of colorectal carcinomas

Anna A. M. van der Wurff, Jan-Willem Arends, Edith P. M. van der Linden, Joop ten Kate, Fred T. Bosman.

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Introduction

The cell adhesion molecule L-CAM, also known as E-cadherin, uvomorulin, Arc-1 or cell CAM 120-80, is a member of the CAM gene family, which plays an important role in epithelial cell-cell interaction[1]. In the process of invasion, loss of intercellular adhesion is one of the early occurring critical steps towards metastasis. Studies on various human and non-human tumour cell lines have revealed an inverse correlation between L-CAM expression and invasive growth. Vleminckx et al.[2] described highly invasive epithelial tumour cells which after transfection with a cDNA encoding for L-CAM, lost their invasive potential. L-CAM expression in normal and cancerous human tissues was described in several reports[3-9]. These revealed L-CAM immunoreactivity in normal human epithelia on the plasma membrane along intercellular borders. Well differentiated carcinomas generally show an L-CAM immunoreactivity pattern comparable to normal tissue, whereas in poorly differentiated carcinomas mostly weak and heterogeneous staining is found. In our previous study on L-CAM expression in colonic carcinomas[10] we also noticed a trend towards decreasing L-CAM expression along with decreasing differentiation. In addition, some adenomas and carcinomas showed apical instead of intercellular staining, suggesting non-functional localization of L-CAM[11]. Our observations indicated, however, that invasive and non-invasive tumours cannot be distinguished on the basis of L-CAM expression.

Extrapolating from the data obtained in in vitro studies on cultured cell lines, which have demonstrated loss of cell adhesion molecules in association with invasiveness[12,13,14,15], one would expect to find reduced L-CAM immunoreactivity in metastases.

To test this hypothesis we analyzed L-CAM immunoreactivity patterns in lymph node and liver metastases originating from colorectal cancer.

Material and methods

Surgical specimens of 12 liver metastases and 10 lymph node metastases were obtained, snap-frozen in isopentane and stored at -70°C until sectioning. Sections of 4 µm thickness were cut, air dried, fixed in methanol for 1 min at -20°C, dipped in acetone at -20°C and stained. To reduce non-specific staining endogeneous peroxidase was inactivated with 0.5 % H_2O_2 in PBS. An indirect immunoperoxidase method was used. Sections were incubated overnight with a monoclonal anti-L-CAM antibody 6F9 (Eurodiagnostica, The Netherlands) in a dilution of 1:40 in PBS-1% BSA at 4°C. The production, specificity and immunohistochemical use of this antibody has been extensively reported by Frixen

et al.[13]. After three washes in PBS, sections were incubated with a rabbit anti-mouse Ig-HRP conjugate (Dakopatts, Denmark) for 1 h at room temperature. After repeated washing in PBS, peroxydase activity was visualized with diaminobenzidine (DAB) and H_2O_2. Finally, the sections were counterstained with haematoxylin and mounted with entellan. As a positive control sections of normal colonic mucosa were used. In this material an intercellular plasma membrane associated pattern of immunoreactivity was consistently shown in colonic mucosal epithelium. As a negative control for each and every section a separate slide was incubated with PBS-1% BSA without the monoclonal antibody or with an irrelevant monoclonal antibody. In these sections consistently no staining was observed.

For microphotography, representative sections were stained using an immunofluorescence method. Sections were incubated with anti-L-CAM antibodies as described above, but after three washes in PBS, they were incubated with biotinylated sheep anti mouse Fab fragments (Amersham, United Kingdom) diluted 1:250 (30 min, room temperature). After repeated washing in PBS, sections were incubated with fluorescein conjugated streptavidin (Dakopatts, Denmark) diluted 1:100 (1 h, room temperature). They were rinsed in PBS, mounted in Immunomount (Shandon, Pittsburgh) and examined with a Biorad MRC 600 Confocal Scanning Laser Microscope, equipped with a mixed Argon/Krypton gas laser. FITC fluorescence was excited with the 488 nm laser line and recorded using a 515 lp band pass filter. The immunofluorescence and immunoperoxidase results were invariably concordant.

Scoring was done by two independent observers (AvdW and JWA). The following nomenclature was used: (1) normal, i.e., a pattern of L-CAM immunoreactivity corresponding with that in normal colon mucosa, which consists of uniform expression along the intercellular borders; (2) apical, i.e., intensified immunoreactivity at the luminal cell surface without intercellular staining pattern; and (3) negative: no immunoreactivity at all.

Results

All lymph node metastases showed L-CAM immunoreactivity, one of them in a predominantly apical, nine in an intercellular pattern. From three tumours we collected two lymph node metastases each (I and II, Table I). In two lymph node pairs the L-CAM staining pattern was identical, in the other pair one lymph node metastasis showed poor differentiation and reduced L-CAM staining with negative areas amounting to thirty percent of the section. No totally negative lymph node metastases were seen. Heterogeneity existed in all lymph node metastases with intercellular staining, apical staining and negative areas in

Figure 1. Intercellular and partly negative L-CAM staining pattern in lymph node metastasis of colorectal carcinoma using an immunofluorescence method with monoclonal anti-L-CAM antibody 6F9.

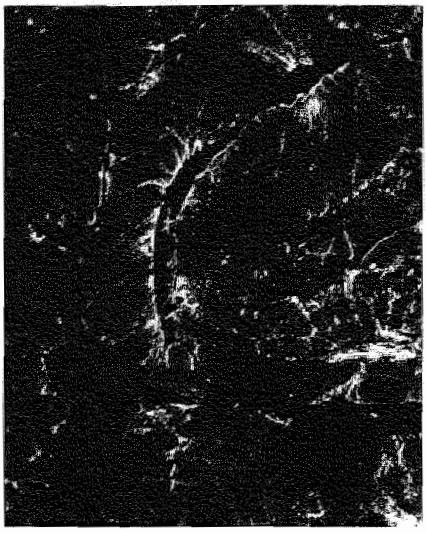

Figure 2. Predominantly apical L-CAM staining pattern in liver metastasis of colorectal carcinoma using an immunofluorescence method with monoclonal anti-L-CAM antibody 6F9.

variable admixtures. Almost all metastases (Fig. 1) showed areas in which an apical as well as an intercellular staining pattern occured (Normal/Apical combined; Table I). Both patterns occurred at random, regardless of local growth pattern or differentiation grade. In three lymph node metastases negative areas accounted for a higher percentage surface area than in the primary tumour. All corresponding primary tumours showed a predominantly normal intercellular L-CAM staining pattern (Table I), with slight heterogeneity in immunoreactivity patterns. Table I also shows the grade of differentiation of the primary tumour. In comparison with the primary tumours the L-CAM staining pattern in lymph node metastases seemed to be somewhat more heterogeneous.
In all liver metastases L-CAM immunoreactivity was demonstrated, in five in a predominantly normal, and in seven in an apical pattern (Table II; Fig. 2). Unfortunately, primary tumours were not available for comparison. No L-CAM negative metastases were seen. Negative areas occasionally accounted for as much as forty percent of the metastatic tumour tissue.

Discussion

L-CAM expression in metastases has not been extensively studied as yet. Schipper et al.[7] reported L-CAM expression in squamous cell carcinomas of head and

Table I. L-CAM expression in lymph node metastases and primary tumours.

	L-CAM positive areas (percentages)									
	Primary tumours					Lymph node metastases				
	Differentiation grade	Normal	Normal/ Apical	Apical	Negative	Differentiation grade	Normal	Normal/ Apical	Apical	Negative
T 3934-92	well	90	5	5	0	moderately	80	10	10	0
T 4010-92	moderately	80	10	10	0	moderately I	70	20	10	0
						moderately II	70	10	20	0
T 8573-92	moderately	90	5	5	0	moderately I	80	10	10	0
						moderately II	80	10	5	5
T 9602-92	moderately	70	10	10	10	poorly I	5	10	55	30
						moderately II	80	10	10	0
T 11423-92	moderately	70	10	20	0	moderately	70	10	10	10
T 11682-92	moderately	100	0	0	0	moderately	100	0	0	0
T 8306-92	poorly	90	5	5	0	poorly	70	15	5	10

Table II. L-CAM expression in liver metastases

		L-CAM positive areas (percentages)			
		Liver metastases			
	Differentiation grade	Normal	Normal/ Apical	Apical	Negative
T 12252-89	well	80	10	10	0
T 519-91	well	10	20	60	10
T 12726-88	well	50	10	30	10
T 4435-91	well	10	5	75	10
T 6833-91	well	40	10	50	0
T 3617-92	well	80	10	10	0
T 4180-88	moderately	5	5	70	20
T 9943-88	moderately	80	10	10	0
T 13514-88	moderately	5	5	60	30
T 955-91	moderately	90	5	5	0
T 1208-92	moderately	40	20	40	0
T 3134-92	poorly	5	10	45	40

neck and their lymph node metastases by immunohistochemistry, immunoblotting and in situ hybridization. Well differentiated carcinomas expressed L-CAM as strongly as normal stratified epithelium, whereas all undifferentiated tumours were L-CAM negative. Also in seven out of eight lymph node metastases no L-CAM immunoreactivity was found. Dorudi et al.[18] used immunohistochemistry and in situ hybridization to assess L-CAM expression in colorectal carcinoma. They also found a relation between L-CAM expression and differentiation grade. In twenty out of 32 lymph node metastases and in seven out of eight liver metastases no L-CAM expression could be found. These findings support the notion that loss of L-CAM expression is a prerequisite for the development of metastasis.

In several other studies L-CAM expression in lymph node metastases is mentioned. The results obtained in adenocarcinomas of the male and female genital tract[8,9] and in the digestive tract[6] indicate that the expression pattern of L-CAM in the primary tumour reoccurs in the same intensity and extent in the metastasis.

Assuming that loss of L-CAM expression is indeed a prerequisite for metastasis, these observations leave at least two possibilities open: emergence of cell clones in the primary tumour with genetic alterations leading to reduced L-CAM expression or, alternatively, temporary down regulation of L-CAM expression during the early phases of the metastatic cascade. In the present report we describe L-CAM immunoreactivity patterns in colorectal carcinoma matastases.
L-CAM immunoreactivity in lymph node and liver metastases was predominantly intercellular or apical, but none of our cases showed total absence of L-CAM immunoreactivity. These results seem at variance with those reported by Dorudi et al.[18]. Methodological differences might account for these discrepancies: Dorudi et al.[18] studied only formalin fixed paraffin embedded material whereas we used unfixed cryostat sections. We have experienced that for the preservation of L-CAM immunoreactivity the duration of formalin fixation and the temperature during fixation are very critical (Dinjens et al., unpublished data). We previously studied L-CAM expression in colorectal adenomas and carcinomas[10]. In line with Dorudi et al.[18] we found a correlation between L-CAM and differentiation.
In lymph node metastases the L-CAM staining pattern was comparable to that seen in the primary tumour although L-CAM negative areas were more prominent than in the primary tumours. In liver metastases more extensive L-CAM negative areas were seen than in lymph node metastases or in primary tumours and a predominantly apical expression pattern was noted. This observation suggests that there might be differences between lymphogenous and haematogenous metastases. We have previously reported loss of expression of tissue antigens in haematogenous metastases, while lymph node metastases showed an antigen expression pattern comparable to that of the primary tumour[16]. An explanation for this phenomenon could lie at the level of selection of subclones of cells differing in antigen composition and metastatic potential[17]. Also tissue specific factors at the site of metastases and different mechanisms leading to lymphogenous or haematogenous metastases could play a role.
In contrast to Schipper et al.[7] we did not observe totally L-CAM negative metastases. Schipper et al.[7] suggest that a constitutive genetic change, such as a point mutation in the cDNA encoding the extracellular domain or in the L-CAM gene promotor, could be responsible for the lack of L-CAM expression[19,20].
Our results, however, suggest a temporary rather than a constitutive L-CAM down regulation at the moment of invasion and L-CAM reexpression at the site of metastases. Tumour cells can only detach from their epithelial environment and traverse the basement membrane into the stroma when cell-cell adhesions are severed. Once settled in their new environment evidently L-CAM is again expressed in order to reestablish tissue architecture. The molecular mechanisms

responsible for this putative transient L-CAM down regulation remain to be elucidated.

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Chapter 6

Patterns of α- and β-catenin and E-cadherin expression in colorectal adenomas and carcinomas

Anna A.M. van der Wurff, Stefan J.T. Vermeulen, Edith P.M. van der Linden, Marc M. Mareel, Fred T. Bosman and Jan-Willem Arends.

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Introduction

In the process of invasion and metastasis, in which cancer cells traverse the basement membrane and reach distant sites via lymph and/or blood stream, cell adhesion plays an important role. Cell adhesion is brought about by molecules which are roughly grouped into four families: Cadherins, Integrins, Selectins and the Immunoglobulin superfamily of cell adhesion molecules[1,2]. E-cadherin, a member of the cadherin family, is expressed in epithelial cells on the plasma membrane along intercellular borders, functions by way of calcium dependent homophilic adhesion[3] and is linked to actin in the cytoskeleton via α-, β- or γ-catenin[4].

E-cadherin expression in cancer cells has been studied extensively. In in vitro as well as in vivo models, suppression of the expression of E-cadherin was shown to induce invasive behaviour[5]. In human cancer tissues, some authors described reduced E-cadherin immunoreactivity with a tendency towards decreased expression along with poor differentiation[6,7](for an overview see Mareel et al.[8]). These observations are in line with results obtained in model systems. It has also become clear, however, that in most invasive and metastatic human tumours E-cadherin is extensively expressed. In a previous study on a series of colorectal adenomas and carcinomas, we found a tendency for E-cadherin expression to be reduced in poorly differentiated carcinomas[9]. Also, the cellular distribution of E-cadherin seemed to be altered in neoplastic cells: in addition to lateral plasma membrane staining, apical staining was noted. A possible explanation for apical E-cadherin staining could be that the molecule is no longer linked to the cytoskeleton and therefore non-functional.

Several studies have shown that for functional cadherin mediated adhesion, the cadherin-catenin complex is crucial[10,11]. Defective cell adhesion occurred in vitro when E-cadherin expression was not paralleled by α-catenin expression[10,12,17]. In functional terms, it is therefore preferable to consider the cadherin-catenin complex rather than its separate components[13-15]. In normal cells E-cadherin and the catenins show an identical pattern of expression. We hypothesized that loss of cell-cell adhesion in invasive cells might be associated with defective E-cadherin and/or a defective catenin expression which might be reflected in discrepancies between the staining patterns for these proteins. To test this hypothesis, we used immunocytochemistry to detect α- and β-catenin expression in normal and neoplastic human colorectal epithelia. The results were compared with previously obtained E-cadherin staining patterns[9,16].

Material and methods

Surgical specimens of 12 colorectal adenomas (1 tubulovillous, 11 tubular) and 13 adenocarcinomas (3 well, 8 moderately well and 2 poorly differentiated adenocarcinomas) were obtained freshly. Normal mucosa was excised from 10 colectomy specimens at a site at least five cm from the neoplasm. Also, 7 lymph node and 6 liver metastases of colorectal adenocarcinomas were sampled. Tissues were snap frozen in isopentane and stored at -70°C until sectioning. Serial sections of 2 µm thickness were cut, fixed in acetone at -20°C for ten minutes, air dried, and stained. The sections were preincubated with normal goat serum in a dilution of 1:5 for ten minutes. An immunofluorescence method was used. Briefly, the sections were incubated with the polyclonal rabbit anti-α-catenin antiserum[17] diluted 1:100 and polyclonal rabbit anti-β-catenin antiserum[18] in a dilution of 1:200 in phosphate buffered saline (PBS)-1% bovine serum albumin (BSA) respectively (one hour,RT) and after three washings in PBS, incubated with biotinylated goat anti-rabbit Fab fragments in a dilution of 1:100 (Nordic, the Netherlands) for thirty minutes. After repeated washing in PBS, sections were incubated with fluorescein-conjugated streptavidin (Dakopatts, Denmark) diluted 1:100 (30 min, RT). For E-cadherin immunostaining, the monoclonal antibody 6F9[19] (Eurodiagnostica, the Netherlands) was used, diluted 1:40 in PBS-1% BSA. Sections were incubated with biotinylated sheep anti-mouse Fab fragments (Amersham, U.K.). Finally, they were rinsed in PBS, mounted in Immunomount (Shandon, Pittsburgh) and examined with a Zeiss LSM 410 confocal laser scanning microscope, equipped with a mixed argon/krypton gas laser. FITC fluorescence was excited with the 488 nm laser line and recorded using a 515 lp band pass filter.

For double staining, frozen sections were fixed and after rinsing in PBS-1% BSA, incubated with the β-catenin polyclonal antibody in a dilution of 1:100 (30 min, RT), followed by an incubation with horse anti-rabbit FITC (Dakopatts, Denmark) (30 min, RT). Then, for E-cadherin staining the sections were subsequently incubated with E-cadherin monoclonal antibody (1:10, 30 min, RT) and rabbit anti-mouse TRITC (1:40, 30 min, RT). The sections were mounted in Vectashield (Vector Laboratories, USA).

In control slides, to check for cross-reactivity, sections with catenin antibody were incubated with rabbit anti-mouse TRITC and sections with E-cadherin antibody were incubated with horse anti-rabbit FITC. Also a section was incubated solely with rabbit anti-mouse TRITC and horse anti-rabbit FITC. No cross reactivity was observed (data not shown). FITC and TRITC fluorescence was excited with the confocal laser scanning microscope and recorded using a dual excitation mode according to the manufacturers specification.

Scoring was performed by two independent observers (AvdW and JWA). The following nomenclature was used: (1) Normal: a uniform expression along intercellular borders. (2) Cytoplasmic: a diffuse cytoplasmic staining pattern. (3) Apical: intensified immunoreactivity at the luminal cell surface without an intercellular staining pattern. (4) Negative: no immunoreactivity at all.
Round percentages of positive staining areas were used as a guideline and are not based on numbers of counted cells.

Results

In normal colon mucosa α- and β-catenin immunoreactivity was found along intercellular borders of all epithelial cells. No immunoreactivity was seen at the basal side facing the basement membrane or at the luminal cell border. No difference was observed in the staining pattern between α- and β-catenin or E-cadherin (Fig.1). Consistently, colocalization of catenins and E-cadherin was found in double labeling experiments. Stromal staining was not found.
In adenomas, α- and β-catenin expression was found at intercellular borders of all epithelial cells in the same intensity as seen in normal colonic mucosa. In addition, weak cytoplasmic staining was noted in most specimens. α-Catenin expression usually appeared to be less intense than β-catenin expression, irrespective of grade of dysplasia. Also, E-cadherin expression showed the same immunoreactivity as in normal mucosa although staining intensity was less pronounced. Again, colocalization of catenins and E-cadherin was consistently found.

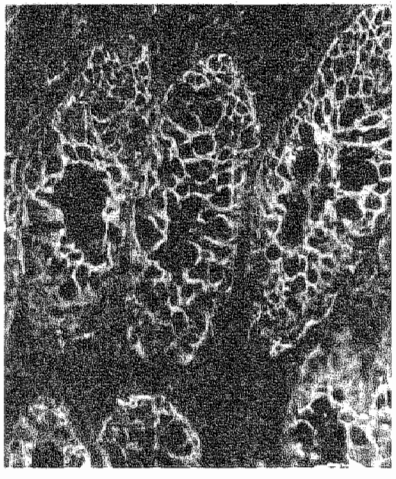

Figure 1. Catenin expression in normal colon mucosa;

In the carcinomas in this series no totally catenin negative adenocarcinomas were encountered. No difference between the α- and β-catenin staining patterns was found. The carcinomas showed intercellular membranous staining, sometimes associated with faint cytoplasmic α- and β-catenin staining, irrespective of differentiation grade. A semiquantitative analysis of staining patterns is presented in Table I. Small negative areas (ten to twenty percent of the total tumour area) were noted in one poorly differentiated and in one moderately differentiated carcinoma. E-cadherin staining was reduced

Table I. Cadherin and catenin expression in carcinomas and lymph node metastases.

	Differentiation grade	Positive area in carcinomas		Positive area in lymph node metastases	
		Cadherin	Catenin	Cadherin	Catenin
T1417-90	Well	80%	100%	nd	nd
T8706-90	Well	100%	100%	nd	nd
T3934-90	Well	100%	100%	100%	100%
T7993-90	Moderate	100%	100%	nd	nd
T8097-90	Moderate	100%	100%	nd	nd
T4010-92	Moderate	100%	100%	100%	100%
T8573-92	Moderate	100%	100%	100%	80%
T9602-92	Moderate	90%	80%	70%	80%
T11423-92	Moderate	100%	100%	90%	80%
T11682-92	Moderate	100%	100%	100%	100%
T1626-90	Moderate	95%	100%	nd	nd
T7384-90	Poor	100%	100%	nd	nd
T8306-92	Poor	100%	90%	90%	100%

nd, not done.

Table II. Cadherin and catenin expression in liver metastases.

	Differentiation grade	Positive area	
		Cadherin	Catenin
T12726-88	Well	90%	100%
T13514-88	Moderate	70%	100%
T4435-91	Well	90%	100%
T519-91	Well	90%	100%
T6833-91	Well	100%	80%
T3617-92	Well	100%	100%

(five to twenty percent of the total tumour area unstained) in one well differentiated and in two moderately differentiated carcinomas. One of these moderately differentiated carcinomas showed small areas of loss of catenin immunore-

Table III. Cadherin and catenin staining (carcinomas and metastases) and differentiation.

Differentiation grade	Number	E-Cadherin		Catenin	
		normal[1]	aberrant	normal[1]	aberrant[2]
Well	9	5	4	8	1
Moderate	14	9	5	10	4
Poor	3	2	1	2	1

1. Normal i.e. an intercellular staining pattern in the total tumour.
2. Aberrant i.e. a negative, apical and/or cytoplasmic staining pattern in various percentages of the total tumour area(see text).

Table IV. Comparison of cadherin and catenin staining in colorectal adenocarcinomas and metastases.

Staining pattern	Number
cadherin = catenin[1]	13
cadherin > catenin[2]	5
cadherin < catenin[3]	8

1. Identical.
2. More cells are cadherin positive than catenin positive.
3. More cells are catenin positive than cadherin positive.

activity occasionally paralleled by loss of cadherin reactivity. For E-cadherin as well as for the catenins, the apical immunoreactivity pattern is noteworthy. In most cases coexpression of E-cadherin and catenins was found (Fig. 2 and Fig. 3), but in a total of four tumours (one well, two moderately and one poorly differentiated) small percentages of tumour areas were noted where catenin staining was not paralleled by E-cadherin staining and vice versa.

All lymph node (Table I) and liver metastases (Table II) displayed membranous α- and β-catenin immunoreactivity, though sometimes weak (Fig. 4 and Fig. 5). In addition, these tissues showed cytoplasmic staining of variable intensity. No significant differences were found in staining pattern or intensity between

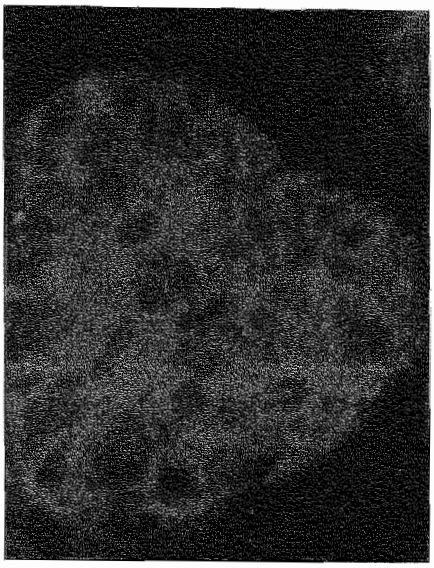

Figure 2. Catenin expression in colorectal adenocarcinoma.

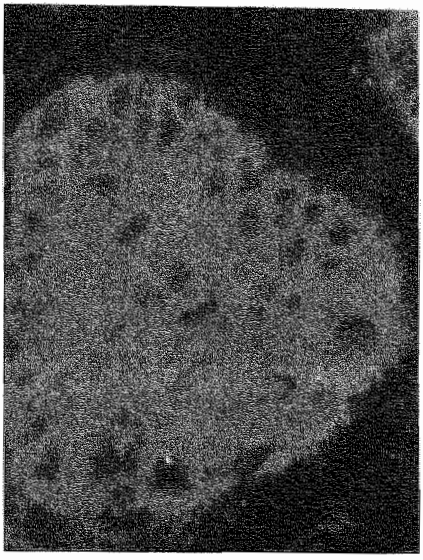

Figure 3. Double staining immunofluorescence for catenin and E-cadherin.

lymph node metastases or primary tumours. Three out of five lymph node metastases from moderately differentiated adenocarcinomas showed in a maximum of twenty percent of the total tumour area lack of α-and β-catenin staining. E-cadherin showed ten to thirty percent negative staining area in two moderately differentiated and one poorly differentiated lymph node metastasis. In both of these moderately differentiated metastases catenin negative areas were occasionally paralleled by loss of cadherin immunoreactivity. In a total of four metastases (from three moderately and one poorly differentiated primary tumour), small percentages of total tumour areas were seen where catenin staining was not paralleled by E-cadherin staining and vice versa.

Of the liver metastases no corresponding primary tumours were available. One well differentiated liver metastasis showed a negative staining area of twenty percent for α- and β-catenin. For E-cadherin, negative staining areas varying from thirty to ten percent were noted in three well and one moderately differentiated liver metastases. In a total of five liver metastases, small percentages of total tumour areas were noted where catenin staining was not paralleled by E-cadherin staining and vice versa. A summary of the data on carcinomas and metastases is presented in Table III. This together with the summary in Table IV reempha-

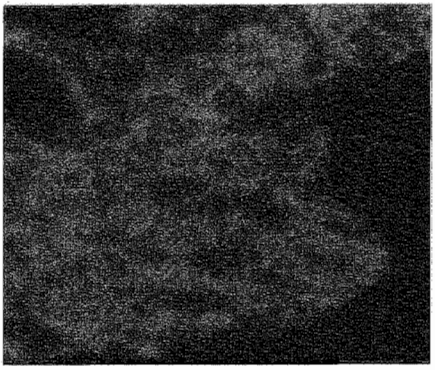

Figure 4. Catenin expression in a lymph node metastasis. E-cadherin showed the same immunoreactivity pattern.

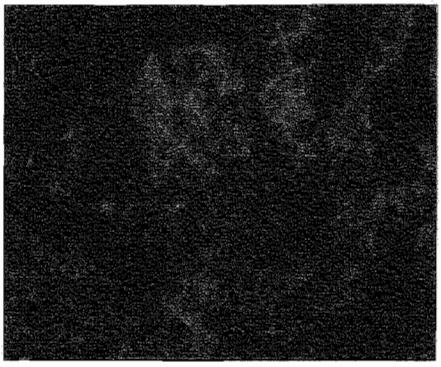

Figure 5. Catenin expresson in a liver metastasis. E-cadherin showed the same immunoreactivity pattern.

sizes our most important observation that in fifty percent of the studied colorectal carcinomas and metastases, E-cadherin and α- and β-catenin expression were identical. When not identical, small percentages of total tumour areas showed catenin staining without E-cadherin staining and vice versa, while α- and β-catenin staining were always identical. Occasionally tumour cells without catenin and cadherin immunoreactivity were seen.

Discussion

Connection of the cytoplasmic domain of cadherin by catenins to the actin filament network of the cytoskeleton is thought to be essential for the adhesive function.

Studies on cell lines indeed showed that E-cadherin function is mediated by catenin[10-12]. In immunohistochemical studies on tissue sections of carcinomas of the human gastro-intestinal tract, including colonic adenocarcinomas[13], reduction of catenin expression was found to be significantly related to poor differentiation, depth of invasion, infiltrative growth and lymph node metastasis[13-15].

In previous studies[9,16] we noted that lymph node metastases of primary tumours expressing E-cadherin showed a comparable E-cadherin immunoreactivity pattern. We therefore postulated that E-cadherin might be transiently downregulated during migratory phases of invasion and we concluded that E-cadherin cannot be used as a prognostic indicator of invasion in colorectal adenocarcinomas. In view of recent publications on the role of the catenins in cadherin function[10-12] we hypothesized that tumours with a normal pattern of E-cadherin immunoreactivity might nevertheless be cell-cell adhesion deficient due to an

ineffective E-cadherin-catenin complex. Disturbance of the E-cadherin-catenin complex by loss of the connection of E-cadherin to the cytoskeleton might also be responsible for the apical E-cadherin expression pattern we previously described in adenomas and carcinomas of the colorectum[9]. We therefore compared α- and β-catenin localization with that of E-cadherin in colorectal adenomas, carcinomas and their lymph node and liver metastases.

In normal colonic mucosa, catenin expression was located on cell membranes along intercellular interfaces, in a pattern identical to that of E-cadherin. In adenomas a corresponding staining pattern was noticed. In only few adenocarcinomas the catenins showed some focal loss of immunoreactivity which was occasionally but not always paralleled by loss of E-cadherin staining. Also, the reverse phenomenon was observed: E-cadherin staining without α- and β-catenin staining. In lymph node and liver metastases similar results were obtained. No difference in staining pattern between α- and β-catenin was found. Therefore, in invasive neoplasms catenin downregulation was not consistent nor did consistent downregulation of E-cadherin immunoreactivity occur. The apical E-cadherin staining pattern was paralleled by an apical catenin pattern.

As a consequence, invasive behaviour of E-cadherin positive tumours can only in few cases in small areas of the tumour be explained by lack of catenins in the E-cadherin-catenin complex. This is in contrast with the results of a study by Takayama et al.[20] in which he concluded that downregulated β-catenin expression is associated with malignant transformation. The difference could be explained by the number of poorly differentiated tumours studied. However in our study of primary tumours and metastases we show that in colorectal cancer invasive and metastatic behaviour is not paralleled by a constitutive decrease in expression of the components of the E-cadherin-catenin complex. An explanation for metastasis in E-cadherin and catenin positive tumours could be transient loss of cell-cell adhesion during invasion as a result of transient downregulation of the expression of E-cadherin as well as of catenins. Alternatively, the observed immunoreactive E-cadherin and catenin could be dysfunctional, for example as a result of gene mutations or through posttranslational modifications. It has been shown that tyrosine phosphorylation of the E-cadherin-catenin complex by v-src or Epidermal Growth Factor (EGF) is associated with loss of cell adhesive properties[21,22]. Of particular interest in colorectal adenocarcinomas is the possibility that the functional state of cell-cell adhesions and their linkage with the cytoskeleton could be altered under influence of a mutated APC gene product. The latter possibility is especially interesting in view of recent publications about binding of the APC oncosuppressor gene product to β-catenin[23,24]. Our observations indicate that in most of colorectal cancers the majority of the cancer cells display a normal (matching) pattern of E-cadherin

and catenin expression. Aberrant expression of the E-cadherin-catenin complex on the plasmamembrane might be inferred from the apical staining pattern which was occasionally noted. Disruption of the E-cadherin-catenin complex was noted both in terms of E-cadherin and/or catenin loss.
In conclusion, matching patterns of E-cadherin and α- and β-catenin staining in normal and most neoplastic colorectal epithelia were found. An incomplete E-cadherin-catenin complex in terms of either decreased E-cadherin staining and/or decreased catenin staining was found in a few cells of a number of adenocarcinomas. These findings exclude the use of E-cadherin or catenin staining of colorectal lesions for diagnostic or prognostic purposes but open interesting new possibilities regarding the functional disturbances of cell adhesion during invasion.

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General discussion

The aim of this study was to identify new prognostic indicators in colorectal adenocarcinoma. The presently available indicators allow relatively useful subdivision of patients according to depth of invasion, lymph node involvement, venous invasion and degree of differentiation. Of these parameters, degree of differentiation is the least defined, as it is based principally on a rather subjective visual impression. The problem with the presently available subdivisions, however, is that they - in spite of significant correlations between pathological characteristics and tumour behaviour at a population level - do not allow individualisation of clinical managment options. The availability of additional criteria to fine-tune prognostic indications would be of tremendous clinical importance.
The approach chosen in this thesis for identification of new prognostic indicators is more or less standard: definition of a new parameter and retrospectively comparing its status in individual cases with tumour behaviour in terms of prognosis, determining its potential usefulness. Unfortunately, most clinicopathological studies focusing on new prognostic markers stop there. This seriously limits a large majority of prognostic studies: a lack of prospective use of a potentially important new parameter as a decisive factor in clinical management of individual patients. An important need in the near future therefore, is the development of prospective trials in which new prognostic markers are used as main determinants for selecting among different availabe patient managment modalities. This holds true not only for colorectal cancer but for almost any tumour type.
Our strategy for the identification of new markers in this study has been defined by prior knowledge regarding the biology of colorectal cancer. We opted for two parallel approaches. Firstly, we decided to look for new parameters related to differentiation, given the fact that differentiation is already in use as parameter but lacks a conceptual basis and clearly defined criteria. Secondly, we decided to study the E-cadherin/catenin family of cell adhesion proteins, knowing that disturbance of cell-cell adhesion plays an important role in the development of invasive growth.
In looking for new markers related to differentiation our working hypothesis was that not the terminally differentiated cells but the immature tumour stem cells are responsible for tumour behaviour because they are clonogenic. Therefore we set out to develop antibodies against immature crypt (stem) cells assum-

ing that these would identify proteins that could function as markers in a more refined classification scheme for colorectal cancer. In order to eliminate common crypt cell antigens during or prior to immunization, we used antibody blocking of common crypt cell antigens and in vivo selection of the immune response by way of cyclophosphamide. This effort resulted in a monoclonal antibody, 5E9, which stains goblet cells in the lower half of the crypt. Upon biochemical characterization 5E9 appeared to recognize a non-sialylated carbohydrate epitope on Muc2. Muc2 is the prominent secretory mucin in the human colon[1]. Mucins in colorectal cancer, either on the cell surface or in the cytoplasm, have not been studied to the same extent as the genetic changes that presumably underlie the development of colorectal cancer. There is sufficient evidence, however, that mucins constitute biologically active molecules involved in the fundamental functional changes that occur in the transformation of an ordinary epithelial cell into a cancer cell and in tumour progression[2]. In a recent study expression of the Muc1, Muc3, TF, Tn and STn mucins and mucin associated glycotopes was examined in colorectal adenomas and carcinomas. Along with tumour development and -progression Muc1, TF, Tn and STn showed increased immunoreactivity along with a shift from apical and/or supranuclear expression to an apolar pattern of expression. In contrast, Muc3 showed unaltered intensity of immunoreactivity but a similar change of pattern towards apolar[2].

These results reinforce the notion that, during carcinogenesis in the colon mucins show characteristic changes of glycosylation and of distribution. A similar study reported that upon malignant transformation expression of Muc1, a non-secretory cell membrane associated glycoprotein, is upregulated and that this accounts for a worse prognosis[3]. It has also been described that high grade atypia in colorectal adenomas is characterized by reduced immunohistochemical expression of Muc2[4]. Furthermore, Muc2 gene expression was found in non-invasive tumours but not in invasive tumours of pancreas and liver[5]. Our Muc2 epitope, identified by 5E9, recognized a subset of Dukes B carcinomas with a trend towards worse prognosis. Our hypothesis is that 1. in the colon the O-glycosylation pattern of Muc2 changes when the crypt cells maturate and migrate upwards to the bowel lumen. 2. that tumour stem cells retain this immature Muc2 epitope and 3. that expression of this epitope is related with a more aggressive tumour cell phenotype. In principle, these hypotheses should lead to additional experiments and such experiments might shed more light on the role of mucins in epithelial cel differentiation in the colorectum and on their use as tumour markers in a clinical context.

Our experiments described in chapter 2 illustrate the difficulties encountered in attempting to generate hybridomas with "tailored" specificity. It is quite conceivable that the use of new methods in biotechnology, such as the phage dis-

play technique, might prove a more promising approach towards the development of new antibodies recognising new potentially useful markers. This consideration has led to experiments in which carefully selected panels of tumour cells or tissues are used to screen phage display libraries for the presence of antibodies recognizing interesting new epitopes.

Our second line of study focused on expression of members of the E-cadherin/catenin complex during colorectal carcinogenesis and tumour progression. The initial working hypothesis was that upon progression towards invasion E-cadherin, a putative invasion suppressor gene, would be no longer expressed either through loss of function, through mutation or through transcriptional down regulation. Our findings, confirming similar studies in the literature which concern a variety of organ systems and tumour types, indicate that in metastatic lesions E-cadherin expression is not constitutively lost (for an overview see Mareel et al.[6]). In developing neoplasms the step towards invasion is probably marked by focal loss of E-cadherin expression but not due to mutational loss, at least in most instances. E-cadherin mutations have been found in a limited number of only some tumour types (most notably lobular cancer of the breast and diffuse type of gastric cancer[7,8]). This implies that E-cadherin expression in the initial phase of expression is temporarily downregulated, only to be reexpressed by the metastatic cells when they have to re-establish tissue architecture in the metastatic lesion.

Meanwhile, the situation has become much more complex with the involvement of the catenins in the E-cadherin/catenin complex. We found overall matching patterns of E-cadherin and α- and β-catenin staining patterns. This finding is compatible with the concept of loss of function of the E-cadherin/catenin complex through decreased expression or loss of function of any one of its components. Mutations of α- and β-catenin have been found[9,10]. In addition, β-catenin function can be abolished through phosphorylation and its phosphorylation has been described by the EGFR, c-erbB-2, c-met and $pp60^{v\text{-}src}$ (for an overview see Ilyas et al.[11]). Interestingly, β-catenin function is also controlled by APC (the gene product of the familial polyposis coli gene), which competes with E-cadherin for β-catenin binding[12,13]. The APC mutations (which according to the latest concept are the earliest genetic lesions in colon carcinogenesis[14]) truncate APC protein, which then can bind but not inactivate β-catenin. This leads to increased cytoplasmic β-catenin. An important signaling function of β-catenin is binding to hTcf-4, a transcription factor which is activated only when complexed to β-catenin[15,16]. It is now assumed that this transcription activation may be one of the earliest steps in colorectal carcinogenesis.

Taken together, these data indicate that patterns of expression of the members of the E-cadherin/catenin complex cannot reliably indicate propensity towards

invasive growth. In a clinicopathological context the correlation of expression with differentiation might be promising. More importantly, the E-cadherin studies have provided new insight in the dynamics of cell adhesion and its role in the process of invasion. The catenin studies have opened up new concepts regarding the role of APC in intracellular signaling and the contributory role of β-catenin.

Few of the goals initially aimed for at the beginning of our studies have been attained. Much has been learned, however, in terms of tumour biology and so indirectly with an impact on clinical aspects of the biology of colorectal cancer. Only through better understanding of the biology of this disease will pathologists be able to develop new parameters to clearly define biological potential of tumours in individual patients. Only through the use of such new descriptors in prospective clinical studies can such parameters be validated.

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Summary

Chapter 1 comprises a general introduction to the field of study and describes the aims of the study.
In **chapter 2**, which is like a material and method paragraph in this thesis, we describe the selection of specific colon crypt cell subpopulations and some immunization protocols. In order to obtain monoclonal antibodies against immature (stem) cells, we tried to eliminate common crypt cell antigens during or prior to immunization by way of in vivo selection of the immune respons using cyclophosphamide and by antibody blocking of common crypt cell antigens. The obtained antibodies are described in short.
The aim of the study described in **chapter 3** was to develop monoclonal antibodies that recognize antigens on immature crypt base cells, on the assumption that in a neoplasm undifferentiated but not the terminally differentiated cells will be responsible for tumour progression. We used colon crypt cells which were isolated from human colonic mucosa by EDTA/EGTA incubation. By stepwise harvesting, crypt base cell enriched fractions were obtained, and after incubation with antibodies against dominant antigens, used as immunogen. Of one crypt base cell specific antibody (5E9), the reactive epitope appeared to be a non-terminal carbohydrate in the mucin O-glycans of the colon. The epitope seemed not to be colon specific, but is expressed in a variety of other tissues. In colorectal carcinomas, 5E9 immunoreactivity identified a subgroup of patients with a tendency for worse prognosis. This study shows that we identified a mucin associated maturation epitope in colonic crypt base cells, of which the expression in colorectal carcinoma stage Dukes B3 may be associated with worse prognosis.
L-CAM, also known as E-cadherin, is a cell adhesion molecule, which is expressed at the intercellular borders of most epithelial cells. In carcinoma cell lines L-CAM has been demonstrated to act as an invasion suppressor. In order to determine whether or not L-CAM expression might distinguish between invasive and non-invasive or metastatic and non-metastatic colon neoplasms, in **chapter 4** we studied L-CAM expression in normal colon mucosa, colon adenomas with various degrees of dysplasia and colon carcinomas by immunohistochemistry, using the 6F9 monoclonal anti L-CAM antibody. Normal mucosa showed evenly distributed distinct L-CAM immunoreactivity along intercellular borders. In adenomas as well as carcinomas a similar though weaker expression

was observed. This pattern showed a trend to decrease in parallel with decreasing differentiation. No correlation, however, was found with Dukes stage or area within the tumour. In some carcinomas L-CAM was expressed at the luminal surface of the cells. In others L-CAM expression was not found.
In **chapter 5** we describe L-CAM expression in lymphogenous and haematogenous metastases of large bowel adenocarcinomas, using an indirect immunoperoxidase method with the monoclonal anti-L-CAM antibody 6F9. All studied metastases- lymphogenous as well as haematogenous -demonstrated L-CAM immunoreactivity in a pattern comparable to that of primary tumours. Intratumour heterogeneity in expression was noted, with normal intercellular, apical (non-functional) and focally negative areas in the same tumour.
The data indicate that primary tumours and their metastases do not differ strikingly in their pattern of L-CAM expression. This would be consistent with transient rather than constitutive down regulation of L-CAM in invasive and metastatic cancer cells.
These results of both studies suggest that L-CAM expression is disregulated or lost as an early event in the development of colon neoplasia and indicate that L-CAM expression does not correlate with invasive or metastatic potential.
Previous in vitro and in vivo model studies have shown that when E-cadherin expression in carcinoma cells is reduced, invasive behaviour ensues. The situation in human cancer in vivo, however, appears to be more complex as immunohistochemically determined E-cadherin expression in various carcinomas, including colorectal cancer, does not always correlate with invasive growth. Loss of cell adhesion during invasion in spite of E-cadherin expression might be associated with a defective cadherin-catenin complex. In **chapter 6** we examined the expression of α- and β-catenin in comparison with E-cadherin in colorectal adenomas and carcinomas and in lymph node and liver metastases.
In normal colon mucosa, α- and β-catenin immunoreactivity occurred along the lateral plasma membrane of the epithelial cells, in a pattern identical to E-cadherin staining. A similar pattern was found in colorectal adenomas and in most malignancies. In general, in neoplastic epithelia, the majority of the cancer cells displayed a normal (matching) pattern of E-cadherin and catenin expression.
We conclude that the patterns of expression of E-cadherin and α- and β-catenin are highly similar in colorectal neoplasms. This observation indicates that invasion in colorectal cancer is not paralleled by consistent loss of expression of the components of the cadherin-catenin complex.

Samenvatting

Hoofdstuk 1 geeft een algemene inleiding en beschrijft het doel van het onderzoek dat in dit proefschrift wordt beschreven. In het kankeronderzoek is men op zoek naar manieren om die gezwellen te herkennen die zich al in een vroeg stadium uitzaaien. Wij hebben ons beziggehouden met kanker van de dikke darm en hebben onderzocht, of het mogelijk is om aan de hand van de aanwezigheid van *ongedifferentieerde cellen* een voorspelling te doen over het gedrag van een tumor (hoofdstuk 3) en of voorspellende waarde kan worden toegekend aan de aanwezigheid van *cadherine* (hoofdstuk 4, 5 en 6).
In **hoofdstuk 2** worden de technieken beschreven die in dit onderzoek gebruikt zijn. De bekleding van de dikke darm - die in plooien (*crypten*) ligt - bestaat uit verschillende celsoorten. Uit deze celsoorten hebben we geprobeerd de zogenaamde *stamcel* te isoleren, die zich onder in de crypt bevindt. In deze stamcellen vinden alle cellen van de darmbekleding hun oorsprong. Isolatie van stamcellen is ons niet gelukt. Wel zijn we erin geslaagd *cryptbodems* te isoleren, waarin zich de stamcel bevindt naast cellen in een vroeg ontwikkelingsstadium (*ongedifferentieerde cellen*).
Tevens beschrijf ik enkele manieren om muizen te gebruiken voor het maken van door ons nader gedefinieerde *antilichamen*. Antilichamen zijn eiwitten, gericht tegen lichaamsvreemde stoffen. Door middel van *kleuring* kunnen antilichamen onder de microscoop de aanwezigheid van dergelijke stoffen zichtbaar maken. Enkele antilichamen die we op die manier hebben verkregen worden in het kort beschreven.
Het doel van het onderzoek dat in **hoofdstuk 3** beschreven wordt, was een antilichaam te ontwikkelen dat ongedifferentieerde cryptcellen herkent. Men neemt namelijk aan, dat in een kankergezwel deze ongedifferentieerde cellen verantwoordelijk zijn voor de groei van de tumor. Dit hoofdstuk beschrijft de kenmerken van zo'n antilichaam (*5E9*). 5E9 bleek een suikergroep te herkennen, die niet alleen op darmcellen voorkomt, maar ook op cellen van andere weefsels. We hebben een groot aantal kwaadaardige dikkedarmgezwellen onderzocht. De resultaten lijken erop te wijzen, dat 5E9 aankleuring geeft van gezwellen van patiënten met een kortere levensverwachting.
Cadherine is een molecule dat zorgt voor de verbinding tussen cellen. In kweken van kwaadaardige cellen is aangetoond dat cadherine voorkomt dat kankercellen zich verspreiden. Om erachter te komen of cadherine het onderscheid maakt

tussen wel en niet uitzaaiende cellen, hebben we in **hoofdstuk 4** de aanwezigheid van cadherine onderzocht in *normaal slijmvlies, goed- en kwaadaardige gezwellen* van de dikke darm. In normaal slijmvlies hebben we cadherine door middel van kleuring aangetoond, gelijkmatig verdeeld langs de wanden waarmee cellen aan elkaar grenzen. In goed- en kwaadaardige gezwellen was de aankleuring gelijk, maar zwakker. Het aankleuringpatroon leek minder duidelijk, naarmate het gezwel minder ontwikkeld was. In sommige gezwellen bleek cadherine voor te komen langs die celwand die geen contact maakt met buurcellen. Andere cellen toonden helemaal geen cadherine-aankleuring.
In **hoofdstuk 5** beschrijf ik cadherine-aankleuring in uitzaaiingen van dikke-darmgezwellen. Al die tumoren toonden eenzelfde aankleuring als die gevonden werd in de oorspronkelijke kanker, hoewel er binnen de tumor gebieden waren die meer of minder sterk aankleurden. Een verklaring hiervoor zou kunnen zijn dat in kankercellen die zich verspreiden en voor uitzaaiingen zorgen, cadherine niet definitief, maar tijdelijk wordt onderdrukt. De resultaten van beide onderzoeken zoals beschreven in de hoofdstukken 4 en 5 suggereren dat cadherinemoleculen vroeg in de ontwikkeling van dikke-darmkanker in aantal afnemen of verdwijnen. Daarnaast lijkt de aanwezigheid van cadherine niet samen te hangen met een neiging tot uitzaaien. Eerdere studies aan laboratorium-modellen hebben aangetoond dat kankercellen gaan uitzaaien als ze minder cadherine bevatten. De situatie in het menselijk lichaam blijkt echter ingewikkelder. We hebben immers gezien dat cadherine in verschillende kankersoorten, waaronder kanker van de dikke darm, aanwezig blijft ondanks een neiging tot uitzaaien. Dit verlies van celbinding zou te maken kunnen hebben met een verstoord cadherine-*catenine* complex. Catenines verzorgen de verbinding tussen de cadherines en het inwendige van de cel. Men heeft aangetoond dat de cadherines hun rol in de celbinding alleen in combinatie met deze catenines kunnen spelen.
In **hoofdstuk 6** hebben wij de aanwezigheid van α- en β-catenine in combinatie met cadherine onderzocht in goed- en kwaadaardige dikke-darmgezwellen en hun uitzaaiingen. In normaal slijmvlies van de dikke darm werd catenine na aankleuren op dezelfde plaats zichtbaar als cadherine. Ditzelfde beeld werd gezien in alle goedaardige en in de meeste kwaadaardige gezwellen. We concluderen dat in het algemeen de kankercellen een overeenkomstige aankleuring van cadherine en catenine vertonen. Dit wijst erop, dat uitzaaiing van kanker van de dikke darm niet vergezeld gaat van een blijvend verlies van cadherines of catenines.

Nawoord

Het proefschrift af op het nawoord na en dat vind ik niet de simpelste opgave. In de afgelopen jaren hebben zóvele mensen een min of meer substantiële bijdrage geleverd en een meer of minder belangrijke rol gespeeld, dat er mij bij het terugdenken steeds meer namen te binnen schieten die een ondoenlijk lange lijst opleveren. Het liefst zou ik ook diegenen met wie ik zo af en toe eens gezellig op de gang kon kletsen noemen in dit nawoord, want deze contacten bepaalden de werksfeer en dus het werkgenot, maar daar ben ik maar niet aan begonnen. Daarom in grote lijnen en in chronologische volgorde zo'n zeven jaren "Maastricht".

De start was een onderzoeksplaats in het Biomedisch Centrum op het lab met *Winand Dinjens*, *Edith van der Linden*, *Annick Haesevoets* en *Marie-Hélène Lenders*. Een betere start had ik mij niet kunnen wensen! Het niet-aflatende enthousiasme en de inspiratie van met name *Winand Dinjens*, *Joop ten Kate* en *Fré Bosman* dienen mij nog steeds als voorbeeld.

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Na de verhuizing naar de nieuwbouw en het Academisch Ziekenhuis kwam ik in opleiding to patholoog. De afstand tussen "research" en diagnostiek was geminimaliseerd, een zeer plezierige bijkomstigheid.
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Curriculum vitae

Anna Adriana Maria van der Wurff werd geboren op 10 maart 1961 in Baarn. In 1979 behaalde zij het eindexamen VWO aan het Baarnsch Lyceum. Gedurende het volgende jaar volgde zij een avondcursus bij de Stichting Aanvullend Onderwijs te Utrecht, waarna zij het Staatsexamen natuurkunde, scheikunde en biologie met goed gevolg aflegde en in september 1980 kon starten met de studie geneeskunde aan de Universiteit van Amsterdam. In 1986 behaalde zij het doctoraalexamen, waarna zij de co-schappen doorliep en uiteindelijk in augustus 1988 het artsexamen behaalde.
In oktober 1988 trad zij in dienst als Assistent-in-opleiding (AIO) aan de Rijksuniversiteit Limburg bij de vakgroep pathologie (prof. dr. F.T. Bosman). In de periode 1991 - 1996 doorliep zij de opleiding tot patholoog (opleider: prof. dr. J-W. Arends). In deze periode was zij van van januari 1993 tot en met november 1994 gedetacheerd in het St. Elisabethziekenhuis in Tilburg (opleider: drs. J.F.M.M. Miseré), waar zij na het voltooien van haar opleiding sinds 1 februari 1996 werkzaam is als klinisch patholoog in een maatschap met drs. J.F.M.M. Miseré, drs. F.H.P.M. van Etten, dr. J.L.J.M. Teepen en dr. C.E.M. Blomjous. Het laboratorium voor pathologie verricht werkzaamheden ten behoeve van het Elisabethziekenhuis, het Tweestedenziekenhuis (vestiging Tilburg en Waalwijk), het Pasteurziekenhuis te Oosterhout, het Bernard Verbeeteninstituut te Tilburg en de huisartsen in de regio Centraal Brabant. Zij is getrouwd met Jan Hadders, neerlandicus en docent aan het Onze-Lieve-Vrouwelyceum te Breda, en is moeder van Tim en Saar.

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